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Total lactic acid bacteria, phenolic compounds and antioxidant activities of pineapple waste and *Indigofera zollingeriana* leaves by liquid fermentation

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ABSTRACT: This study aims to determine the best combination of pineapple waste liquid fermentation and *Indigofera zollingeriana* leaves, which produces the highest amount of lactic acid bacteria, the highest vitamin C content, and total phenols, and the best antioxidant from the combination of the fermentation results. The resulting fermentation product will be used as a natural feed additive in poultry rations. This research was conducted with an experimental method using a Completely Randomized Design (CRD), which consisted of five preparations and four replications. The treatments were combination of pineapple waste and *Indigofera zollingeriana* leaves: P1 (100% pineapple waste), P2 (98% pineapple waste and 2% *Indigofera zollingeriana* leaves), P3 (96% pineapple waste and 4% *Indigofera zollingeriana* leaves), P4 (94% pineapple waste and 6% *Indigofera zollingeriana* leaves) and P5 (92% pineapple waste and 8% *Indigofera zollingeriana* leaves). Substrate from each combination of pineapple waste and leaves of *Indigofera zollingeriana* was fermented with the contribution of 10% of lactic acid bacteria obtained from commercial yogurt and incubated for 72 hours. The parameters in this study consisted of total lactic acid (*Lactobacillus bulgarius*), the concentration of vitamin C, total phenols, and antioxidant activity. The results showed that the combination of pineapple waste and *Indigofera zollingeriana* leaves proved significantly ($P < 0.05$) to total lactic acid bacteria (*Lactobacillus bulgarius*), vitamin C concentration, total phenols and antioxidant activity of the fermented liquid (supernatant) products. In conclusion, the combination of 92% pineapple waste and 8% leaves of *Indigofera zollingeriana* had the highest total bacterial contribution, produced the highest vitamin C and phenolic compounds, and increased antioxidants.

Keywords: antioxidant activity; *Indigofera zollingeriana* leaves; pineapple waste

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INTRODUCTION

The livestock industry is a fast-growing industry in Indonesia because of the steady growth of poultry meat consumption among people. The increasing need for meat encourages farmers to pay more attention to the quality of products produced and maintain the health status of livestock that are kept. One way that can be done to improve product quality is by adding natural feed additives to animal feed. Natural feed additives generally come from plant ingredients that contain certain active compounds, both as antioxidants and natural antibiotics. The addition of organic acids in drinking water or feed for broiler proved to be able to increase absorption by increasing the function of digestive enzymes so as affect digestion and absorption, especially fiber and protein (Atapattu and Nellisgaswatta, 2005; Abdel-Fattah *et al*., 2008).

One way to produce organic acids is to use pineapple waste and leaves of fermented *Indigofera zollingeriana* by utilizing the *lactobacillus* bacteria contained in yogurt, the use of yogurt is useful as a source of lactic acid bacteria. In the fermentation process, besides requiring a source of bacteria from yogurt, it also requires a substrate for bacterial growth. A substrate in the fermentation process can be produced from pineapple waste, where pineapple waste contains food substances such as protein, glucose, and fructose (Andriani *et al*., 2013). Apart from pineapple waste, the use of *Indigofera zollingeriana* leaves can also be used as a substrate. Palupi *et al*. (2014) *Indigofera zollingeriana* leaves contain 28.98% crude protein and relatively low NPN, which is 2% so that the leaf protein of *Indigofera zollingeriana* can be relied upon as a source of Nitrogen. Nitrogen is needed because it can accelerate the growth of *lactobacillus* bacteria in fermentation.

Fermented fruits and vegetables are known to produce probiotic drinks that contain phenol compounds and have antioxidant functions. Sutedjo (2015) reported that the addition of star fruit 20% with 8 hours fermentation time resulted in vitamin C of 0.022% and the total phenol compound of 1.533% with antioxidant activity of 50.313%. The results of Widagdha's research (2015) reported that yogurt fermented drinks added by 20% grape juice with 12 hours fermentation time had a total phenolic compound of 1.17% with antioxidant activity of 56.475%. Research on the combination of pineapple waste and leaves of *Indigofera zollingeriana* fermented by the addition of lactic acid bacteria by the utilization of *lactobacillus* bacteria in yogurt has never been done. Fermented products will be used as acidifiers in poultry diets.

MATERIALS AND METHODS

Research material

The materials or ingredients used in liquid fermentation were pineapple waste, *Indigofera zollingeriana* leaves, aquades, granulated sugar, Cimory plain yogurt. While the materials used for the analysis of organic acids were aquades, acetic acid, lactic acid, citric acid, 85% phosphoric acid, KH_2PO_4. Additionally, the ingredients used for antioxidant testing were supernatant from fermented pineapple waste and leaves of *Indigofera zollingeriana*, aquades, DPPH, 5% Na_2Co_3 solution, and methanol.

The tools used in liquid fermentation were a plastic bag, blender, scale, stove, steamer pot, 20 units of 3-liter-capacity plastic jars, paper labels, wooden spoon, and sticky tape. While the tools used for the analysis of organic acids using the HPLC method were HPLC (*High-Performance Liquid Chromatograph*) tool, pipette, aluminum foil, spoon, magnetic stirrer, 0.2 and 0.45 μm *Whatman* filter paper, plates, desiccator, cup clamp, analytic balance. Furthermore, the tools used for antioxidant testing were s*pectrophotometer*, cuvette, vortex, beaker glass, stirring rods, test tube, and rack.

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Research method

The study was conducted with an experimental method using a Completely Randomized Design (CRD), which consisted of five treatments and four replications. The treatment in this study was a combination of pineapple waste and *Indigofera zollingeriana* leaves. The combination of these treatments are:

P1: 100% fermented fresh pineapple waste
P2: 98% fermented pineapple waste with 2% *Indigofera zollingeriana* leaves
P3: 96% fermented pineapple waste with 4% *Indigofera zollingeriana* leaves
P4: 94% fermented pineapple waste with 6% *Indigofera zollingeriana* leaves
P5: 92% fermented pineapple waste with 8% *Indigofera zollingeriana* leaves

Research conduction procedure

Pineapple waste fermentation process referred to Nurhayati *et al*. (2014) was modified using *Indigofera zollingeriana* leaves. Fresh pineapple waste that had been cleaned and fresh leaves of *Indigofera zollingeriana* were chopped, then weighed based on the arrangement and then put into a plastic bag. Subsequently, the plastic bag with materials follows the steam process for 30 minutes intended for sterilization. Then, the process was carried out for 10 minutes before, then put into a jar, the fermentation process by adding water to the substrate involving 1:2 w/v, 15 grams of sugar, and yogurt as much as 100 ml/kg. The fermentation incubation time was 72 hours.

During fermentation, an everyday stir was carried out on the fermentation media, and this aimed to homogenize the nutrients in the fermentation media. Next, the separation between the supernatant and the fermented biomass was carried out. Then, the amount of waste biomass of pineapple and leaves of *Indigofera zollingeriana* formed, and the amount of supernatant containing organic acids was determined after the fermentation process took place. The fermented supernatant was followed by an analysis of the degree of acidity, total *lactic acid bacteria*, total acid concentration, total phenolic compounds, and antioxidant activity.

Observed variables

1. Lactic Acid Bacteria Number Test

The LAB test followed the procedure of Hidayat *et al*. (2013). The plate count method (Total Plate Count) was used to determine the total LAB. The calculation of total LAB was calculated on the planting of Man Rogasa and Sharpe (MRS) media. Calculation of total LAB began with samples diluted in sterile aquades in a ratio of 1:9. Dilutions were carried out from 10^{-1} to 10^{-7}. The cup making was done using MRS agar media. Making MRS of 65.13 g was dissolved in 1000 ml of distilled water, then dissolved. MRS was a water bath at 95^0C until it dissolved and sterilized at 121^0C for 15 minutes.

Preparation of the cup media was carried out with 1 ml of the dilution sample inserted into a Petri dish that already contained MRS agar. The cup making was carried out from a 10^{-6} to 10^{-7} dilution. Then the cup was moved to form the number 8 so that it was homogeneous. Once stable, the cup was incubated in an upside-down position at 37^0C for 48 hours and counted the growing colonies using Colony Counter. The total number of colonies counted must meet the ICMF standard, which was between 30 - 300 colonies per Petri dish.

2. The concentration of Vitamin C Test

The sample was weighed as much as 10-30 grams, then put into a 100 ml volumetric flask and then added distilled water to the boundary mark. Then the filtrate is homogenized and filtered with filter paper. From the obtained filter, 25 ml was taken and put into 100 ml Erlenmeyer, then, 1 ml of 1% starch was added to it. The filtrate that was added with starch was titrated with a standard 0.01 N iodine solution until a color change occurred. Vitamin C levels were calculated using the following formula:

Vitamin C (%) = (ml iodine x 0.01 N x 100/25 x 88 x 100)/weight of material (mg).

3. Total Phenol Compounds Determination

0.4 mL sample adds into a 10 ml measuring flask. Then, 0.4 mL Folin-Ciocalteu reagent add and well-shaken. After five minutes, 4 mL of 7% Na_2CO_3 was mixed with aquades until it reached a volume of 10 mL. Incubation was performed for 90 minutes at 23°C then absorbance readings were carried out using a spectrophotometer at λ 750 nm (Lee *et al*., 2003).

4. Antioxidant Activity

The antioxidant activity test used DPPH (*Diphenyl Pikril hidrazil*) method, according to AOAC (2005), was as follows: Samples were taken as much as 1 ml. The sample solution was made into four series of dilutions, which were 0, 5, 10, and 15. The sample solution for each dilution was taken from 1 ml, then added 9 ml of methanol to the test tube and homogenized.

Each dilution series was taken 2 ml, and then 2 ml DPPH solution was added and homogenized with a vortex. For the preparation of the DPPH solution, 2 ml of DPPH was taken and dissolved using 2 ml of methanol into a 50 ml measuring flask. DPPH solution was put into a cuvette and then absorbed by spectrophotometer (wavelength 517 nm) and recorded as absorbance blank. The vortex solution was left in a dark room for 30 minutes then put into a cuvette, and the absorbance value was measured with a spectrophotometer (wavelength 517 nm) and recorded as absorbance of the sample. The formulas used to calculate antioxidant activity are:

$$\% \text{ Inhibition} = \frac{(\text{A control} - \text{A sample}) \times 100\%}{\text{A control}}$$

Note: A control = Absorbance does not contain samples

A sample = Sample absorbance

Data analysis

Data analysis uses the analysis of variance following the Completely Randomized Design. If there are significant differences in treatment, further tests will use the Duncan Multiple Range Test (Steel and Torrie, 1991).

RESULTS AND DISCUSSION

Effect of treatment on the content of lactic acid bacteria

Lactic acid bacteria observed in liquid products fermented from pineapple waste and *Indigofera zollingriana* leaves in this study were types of *Lactobacillus bulgarius* bacteria. The average number of lactic acid bacteria from the combination of pineapple waste and *Indigofera zollingriana* leaves during fermentation are shown in Table 1. Based on the average number of Lactobacillus bulgarius bacteria in Table 1, it can be seen that the combination of pineapple waste and leaves of *Indigofera zollingeriana* influenced the content of lactic acid bacteria fermented. The increasing use of *Indigofera zollingeriana* leaves in combination with the substrate, further increased the population of the lactic acid bacteria. *Indigofera zollingeriana* leaf is one of the legume plants that has a high crude protein content, which is 28.98% and its low NPN content is 2% (Palupi *et al*., 2014) so that the leaves of *Indigofera zollingeriana* have the potential to be a source of Nitrogen for growth lactic acid bacteria during the fermentation process. Okafor (2007) stated that lactic acid bacteria require a carbon source of around 46-52% and a nitrogen source of 10-14% and minerals for their growth.

Table 1. Average of *Lactobacillus bulgaricus* bacteria of liquid fermented products from pineapple waste and *Indigofera zollingeriana* leaves.

Treatment	Amount of *Lactobacillus bulgaricus* bacteria (CFU/mL)
P1	$6{,}5 \times 10^4$
P2	$9{,}1 \times 10^6$
P3	$1{,}7 \times 10^7$
P4	$8{,}4 \times 10^8$
P5	$2{,}3 \times 10^8$

Winarsih (2005) stated that not all bacteria could be used as probiotics. Several requirements must be fulfilled, including having antimicrobial and anti-carcinogenic activity, being able to colonize the digestive tract, and being able to increase intestinal absorption. Some types of probiotics that are often used are *Bifidobacterium brevis*, *B. infantis*, *B. longu*, *Lactobacillus acidopholus*, *L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *L. casei*, and *Streptococcus thermophilus*. This probiotic product is marketed or sold in the form of milk and food supplements.

The results of this study are in line with some of the results of research on fermentation of pineapple juice production and the manufacture of probiotic drinks, including the results of research by Elsaputra *et al*. (2016) that pineapple skin fermentation produced the content of lactic acid bacteria 7.08 x 107 cells/ml with a pH of 3.94. Tambunan's research results (2016) stated that the content of lactic acid bacteria of pineapple juice after fermentation was 1.1 x 108 CFU/ml, with a pH of 5.67. Rizal *et al*. (2016) state that the best lactic acid bacterial strain is *Lactobacillus casei,* which produces a pH of 3.54 with a bacterial amount of 1.1 x 1010 CFU/ml. Then Angrestian *et al*. (2014) reported that the initial population of lactobacillus bacteria in the manufacture of antibiotic drinks was 6.5 x 104 CFU/ml and increased to 9.8 x 108 CFU/ml.

Treatment effect on the concentration of vitamin C and phenol compounds

The average vitamin concentration of liquid product fermented from pineapple waste and leaves of *Indigofera zollingriana* are shown in Table 2.

Table 2. The average concentration of vitamin C and phenolic compounds of liquid fermented products from pineapple waste and *Indigofera zollingeriana* leaves.

Treatment	Vitamin C Concentration (%)	Total Phenolic Compounds
P1	$0{,}10 \pm 0{,}01^a$	$1{,}95 \pm 0{,}31^a$
P2	$0{,}20 \pm 0{,}05^a$	$3{,}15 \pm 0{,}35^b$
P2	$0{,}66 \pm 0{,}07^b$	$3{,}03 \pm 0{,}48^b$
P4	$0{,}69 \pm 0{,}07^b$	$3{,}93 \pm 0{,}23^c$
P5	$0{,}66 \pm 0{,}07^b$	$4{,}18 \pm 0{,}10^c$

Note: Numbers followed by different letters in the same column show significant differences ($P < 0{,}05$).

The results of the analysis of variance showed that the combination of pineapple waste and leaves of *Indigofera zollingeriana* had a significant effect ($P < 0.05$) on the concentration of vitamin C of fermented liquid products (supernatants). The significant effect happens because of the higher content of *Indigoferaa zollingeriana* leaves improves the population of lactic acid bacteria in the fermentation results. Concerning the increase in *Lactobacillus bulgaricus* bacteria with vitamin C content, it can be seen that the bacteria produce acidic conditions that could increase the level of vitamin C itself. The phenomenon is

similar to the opinion of Gaman and Sherrington (1994), that cooked fruits will lose less vitamin C than vegetables because their presence is more acidic so that the speed of oxidation is reduced. Also, the sour taste caused by *Lactobacillus bulgaricus* activity is possible for the presence of vitamin C.

Based on the results of further tests of treatments, P3, P4, and P5 showed that the treatment containing the highest content of vitamin C was due to the increased use of leaves of *Indigofera zollingeriana*. While treatment P1 and P2 contain lower content of vitamin C, because the population of *Lactobacillus* bacteria in the treatment is also lower, so the amount of lactic acid produced is less than the treatments of P3, P4, and P5.

The results of Silalahi's research (2009) reported that *Lactobacillus bulgaricus* had been known to play an essential role in producing high lactic acid in the manufacture of fruitghurt. The principle of making fruitghurt is fruit fermentation using bacteria. Good fruitghurt has a total lactic acid of about 0.85 -0.89% and a degree of acidity (pH) of around 4.5. Sutedja and Nisa (2015) reported that the addition of star fruit in making yogurt increased the total acid and vitamin C content of the yogurt produced. Based on the analysis of variance on the total concentration of phenol compounds in liquid products that the combination of pineapple waste and *Indigofera zollingeriana* leaves had a significant effect (P <0.05) on the total phenol compounds produced because of the increasing use of *Indigofera zollingeriana* leaves in the substrate that increased the number of lactic acid bacteria in the fermentation product, thus increasing the change in carbohydrate substrate by these bacteria and increasing phenol compounds produced at the end of fermentation.

The fermentation process, by the addition of *Saccharomyces sereviceae* inoculants in the substrate, increases the phenol compounds produced (Asngat *et al*., 2011; Kunaepah, 2008). Pineapple waste fermentation results with *Saccharomyces sereviceae* as much as producing a total phenol compound of 3.7% (Melani, 2012)

Effect of treatment on antioxidant activity

The average antioxidant activity of liquid fermented products from pineapple waste and *Indigofera zollingeriana* leaves is shown in Table 3.

Table 3. The Average of antioxidant activity of liquid fermented products from pineapple waste and *Indigofera zollingeriana* leaves.

Treatment	Antioxidant Activity (%)
P1	51,77±0,55[b]
P2	51,60±3,59[b]
P3	47,71±1,71[a]
P4	48,29±0,03[a]
P5	45,37±1,34[a]

Note: Numbers followed by different letters in the same column show significant differences (P <0,05)

Antioxidants found in pineapple skin fibers included in the group of polyphenol compounds are antioxidants that have several phenol functional groups. This type of antioxidant prevents the oxidation process through the mechanism of capturing free radicals. Thus, the concentration of oxidants and antioxidants in the body remains balanced (Mahyanti, 2007). Then the antioxidant of the liquid product also comes from *beta-carotene,* which is found in the leaves of *Indigofera zollingeriana*. Palupi *et al*. (2014) reported that Indigofera sp. contains beta-carotene compounds of 507.8 mg/kg, which is a source of antioxidants. In addition to *beta-*

carotene, *Indigofera zollingeriana* leaves also contain tannins. Tannins are water-soluble phenolic compounds, which come from vascular plants with molecular weights of 500 to 3000 grams/mol.

These compounds are widely distributed in leaves, fruit, bark, and stems, generally taste astringent. Tannins have biological activities as chelating metal ions, biological antioxidants, and are antibacterial compounds (Suwandi, 2012). The increasing proportion of *Indigofera zollingeriana* leaves in the fermented substrate increased the antioxidant activity of the fermented liquid product because of the higher carbohydrate breakdown process by lactic acid bacteria into phenol compounds in the fermentation product. In line with Hurr *et al*. (2014) that the increased concentration of phenol compounds causes an increase in antioxidant activity.

The antioxidant activity produced in the fermentation products of pineapple and *Indigofera zollingeriana* leaves is caused by probiotic bacteria producing lactic acid. Lactic acid contains α-hydroxy acids (AHA) that function as antioxidants and are often used for the manufacture of cosmetics and in food products (Yu and Van Scott, 2002). Apart from lactic acid, which is the main result of metabolism, probiotic bacteria also produce compounds that act as antioxidants.

These antioxidant compounds are secondary metabolites produced by probiotic bacteria.

CONCLUSION

Based on the results of the study, as the use of *Indigofera zollingeriana* leaves in a combination of pineapple waste substrate increased, the more the population of lactic acid bacteria, the concentration of vitamin C, the total phenolic compounds of liquid fermented products that contained pineapple waste and *Indigofera zollingeriana* leaves increased, as well as antioxidant content of the product that is seen from the increasingly decreased antioxidant activity in a combination of 92% pineapple waste and 2% *Indigofera zollingeriana* leaves, which amounted to 45.37%.

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