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In Vitro Inhibition Test of Turmeric and in Vivo Total Bacterial Count in the Intestinal Digesta of Mojosari Duck Fed Diet Containing Graded Levels of Turmeric

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Abstract

This study aimed to determine the inhibition value of turmeric (Curcuma longa) against Salmonella and Escherichia coli and evaluate the bacterial population in the small intestinal digesta of Mojosari ducks. This study was arranged using a Completely Randomized Design (CRD) with six treatments and four replications; eachreplication consisted of eight Mojosari ducks.The in vitro study used four treatments of different concentrations of turmeric, consisting of Control/only water (C0), turmeric 98% + 2% water(C1), Probiotic 98% and 2% water (C2), turmeric 92%, and probiotic 8% (C3),and Bacitracin 92% + water 8% (C4). Then, the inhibition zone diameter was measured using the agar well diffusion method. On the other hand, the in vivo study was carried out by employing 192 Mojosari duck raised for 60 days. They were given the following dietary treatments, namely T0= basal diet, T1= basal diet supplemented with turmeric 0.2%, T2= basal diet supplemented with supplemented with turmeric 0.8%, T3= basal diet supplemented with turmeric 0.2%+probiotic 0.1% and T4=feed basal supplemented with supplemented with turmeric 0.8% + probiotic 0.6%, and T5 = basal diet + Zinc Bacitracin 0.01%. The variables measured were the total bacterial population based on the Total Plate Count (TPC) in the small intestinal digesta of Mojosari ducks. The Data were analyzed using one-way ANOVA. The differences among means were tested using theLeast Significant Difference (LSD) test.

Introduction

Mojosari duck is local germplasm that can potentially contribute animal protein such as eggs to Indonesian people, especially in rural areas (Henrik and Marhayani, 2020; Hidanah et al., 2018). The use of feed additives in poultry farming in the form of antibiotics has generally been carried out to spur growth or increase poultry productivity and feed efficiency (Lestari et al., 2021; Sjofjan and Adli, 2020). Feed additives of this type often have a negative impact on human health (Landers et al., 2012; Putu, 2009). The European Union community has imposed a practical ban on the use of antibiotics in animal feed since January 1, 2006 (based on regulation number 1831/2003), while in Indonesia, it was implemented on January 1, 2018 (based on the regulation of the Ministry of Agriculture number 14 of 2017). Bioactive substances contained in plants or herbal extracts have been widely studied to replace synthetic antibiotics (Diaz-Sanchez et al., 2015; Toghyani et al., 2011)

Natural antibiotics can be found in several tuber plants, including turmeric (Curcuma longa). Turmeric components consist of curcumin (73.4%), desmeto curcumin (16.1%) and bisdesmeto

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curcumin (10.5%). Curcumin can function as a cholagogum (stimulates the gallbladder walls, which play a role in the fat breakdown), hypolipidemic, hepatoprotector (protects the liver from toxins), and improves blood circulation. The chemical content of turmeric essential oil consists of ar-tumerone, and -tumerone, tumerol, -atlanton, -caryophyllene, linalol, 1,8 cineol. Previous research has also proven that turmeric powder in broiler feed could act as an immunomodulatory by increasing the phagocytic activity of polymorphonuclear cells (PMN), challenged with Escherichia Coli bacteria in vitro (Antony et al., 1999). Turmeric rhizome has pharmacological effects such as antimicrobial, antioxidant, anti-inflammatory, and improve the work of the digestive organs and reduce strong bowel movements to prevent diarrheal disease in poultry. The result study using turmeric extract at a concentration of 50 ml was able to inhibit Escherichia coli bacteria's activity. The inhibited activity of Escherichia coli bacteria could increase the production of non-pathogenic bacteria, thereby improving the balance of bacteria or intestinal microflora in poultry. This study aims to determine the levels of curcumin, flavonoids, and polyphenols and the diameter of the inhibition zone of turmeric against Salmonella and Escherichia coli bacteria in the digestive tract of ducks.

Methods

The isolation of the active compound of turmeric was carried out at the Phytopharmacy Laboratory of the Faculty of Pharmacy, Hasanuddin University Makassar. Meanwhile, the inhibition test of turmeric against Salmonella and *Escherichia coli*was carried out at the Laboratory of Plant Pests and Diseases, Faculty of Agriculture, Universitas Brawijaya.

Preparation of turmeric extract

The material used is turmeric rhizome which is dried for seven days in temperature room and then mashed. The extraction process was started by weighing 250 g of turmeric flour, then soaked in 1250 ml of 95% ethanol and left for two days while stirring 5-15 minutes every day. The solution was then filtered to separate the filtrate and residue. The resulting residue was soaked again with a 10 ml ethanol solution with an ethanol concentration of 50 ppm - 100 ppm and then filtered again. The filtered filtrate I and II were mixed and evaporated using a vacuum evaporator, then dried in an oven for three days. The inhibition test was conducted using the Kirby-Baeur method (Zada, 2021). The evaporation results were divided into four concentrations by mixing distilled water, consisting of 2%, 4%, 6%, and 8%, and were formulated with various percentages of turmeric.

Research in vitro inhibition test of the effect of adding turmeric extract to bacteria

The preparation of the ingredients begins with preparing the turmeric herbal extract. Then sterilize the tools and test media for Mueller Hinton Agar (MHA) bacteria using an autoclave at 121 ^{0}C for 30 minutes. Furthermore, the diameter of the inhibition zone of *Escherichia Coli andSalmonella shigella* bacteria was tested using the agar well diffusion method.

The further step is to follow the working procedure of the inhibition zone diameter test and continue with the inhibition zone diameter test.The steps of the inhibition zone diameter test: 1) Prepare each of the bacteria *Escherichia coli*and Salmonella cultured as 10^8cfu/ml. The media used for bacterial growth were Eosin Methylene Blue Agar (EMBA) and *Salmonella shigella* Agar (SSA) media; 2) Put the diluted EMBA media into a 1 mm thick petri dish; 3) Using a micropipette, the cultured *Escherichia coli* bacteria were put into a sterile petri dish as much as 1 ml; 4) The petri dish is closed to avoid contamination, then shaken according to the number eight pattern so that the EMBA media and *Escherichia coli* bacteria are evenly mixed. Furthermore, the petri dish can stand for 3-5 minutes so that the EMBA media hardens. After hardening, one well was made in the middle of the petri dish using a sterile blue tip with a 0.7-

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0.8 cm diameter and one well for one treatment; 5) Each treatment was dripped one drop into each well using a 1 ml micropipette. Dilution using ethanol according to each treatment C0, C1, C2, C3, C4. After being mixed, they were stirred to mix the dilution; 6) Petri dishes containing EMBA media, each treatment of *Escherichia coli* and Salmonella bacteria, were incubated in an incubator for 24 hours at 37^{o}C; 7) After seeing the zone of inhibition on the petri dish, then measure it using a ruler (cm) using the Kirby-Baeur method, namely: a) The radical zone, which is the measurement area, did not find any bacterial growth. The antibacterial potential was measured by measuring the diameter of the radical zone (Davis and Stout, 1971); b) Irradical zone, which is an area where bacterial growth is inhibited by antibacterial but not killed (Davis and Stout, 1971); 8) Measurement of diameter is carried out by measuring the diameter of the inhibition zone, including the horizontally and vertically wells, after which it is averaged. The average diameter of the wells reduces the average yield, then the diameter of the inhibition zone of *Escherichia coli* and *Salmonella sp* bacteria is obtained.

The same procedure was applied to the Salmonella bacteria test, but the medium used for bacterial growth was *Salmonella shigella* Agar (SSA).

Biological Research: addition of turmeric flour and probiotics to the gut microflora of Mojosari ducks

This research was carried out by keeping Mojosari ducks for 2 (two) months and feeding them according to treatment. Furthermore, each experimental unit was taken one duck sample and then slaughtered. Before the slaughter process is carried out, hands and equipment must be sterile so that the surrounding bacteria do not multiply by spraying alcohol. After slaughtering the ducks, the digestive organs, including the jejunum, ileum, and cecum, were immediately put into each treatment box that had been coded and contained 10% Buffered Neutral Formalin (BNF). Then the samples were brought to the microbiology laboratory to be tested for the bacterial population using the TPC (selective medium) method, referring to the Thermo Scientific method (Fradiaz, 1993; Jaengkarnkit, 2019)

The procedure for determining the Total Plate Count (TPC) is divided into two stages:

Analysis stage

The sample was weighed as much as 1 gram aseptically, then crushed using a sterile motor then added 9 ml of sterile BPW solution and dovortex to make it homogeneous. Furthermore, transfer 1 ml of the suspension using a sterile pipette into the BPW solution to obtain a 10-2 dilution. In the same way, further dilutions were carried out according to the estimated number of bacteria in the sample up to a dilution of 10-9. Then 0.1 ml of the sample from each dilution was put into a petri dish containing selective PCA media then spread using a sterile cotton swab and performed in duplicate for each dilution of 10-6, 10-7, and 10-8. Subsequently, 0.1 ml of the sample from each dilution above was put into a petri dish containing SSA, EMBA, and MRSA media, then spread using a sterile cotton swab and performed in duplicate for each 10-7 dilution. The petri dish containing the sample and fertilization media was incubated at 35 – 37oC for 24-28 hours for SSA, EMBA, and PCA media. Meanwhile, the suspensions grown on MRSA media were incubated under anaerobic conditions and then incubated at 42^{o}C for 24-28 hours.

TPC calculation stage

Calculation of the Total Plate Count (ALT) or Total Plate Count (TPC). The calculation of ALT or TPC refers to the Indonesian National Standard (2000) concerning the Maximum Limit

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of Microbial Contamination and the Maximum Limit of Residues in Food Ingredients of Animal Origin. Total plate count is a method of counting colonies that grow on NA media. The number of bacterial colonies counted on a petri dish is between 25-250 colonies. After that, the amount obtained is multiplied by the dilution.

$$TPC = \frac{\text{Number of bacteria in a petri dish} \times 1}{\text{dilution factor}}$$

Research Design

The method used is a laboratory experiment to test the inhibition of turmeric against bacteria using a completely randomized design (CRD) with five treatments and four replications. Research treatments include ; C0 = Aquades (Control); C1= turmeric 98%+ aquadest 2% ; C2= probiotic 98% + aquadest 2%; C3= turmeric 92% + probiotic 8%; C4= Bazitracin 92% + aquadest 8%)

The method used was the intestinal microflora of Mojosari ducks using a completely randomized design (CRD) with 6 treatments and 4 replications, one experimental unit consisted of eight ducks. The research treatments included: T0= Basal diet + 0% control; T1= Basaldiet + 0.2% turmeric flour; T2= Basal diet+ 0.8% turmeric flour; T3= Basal diet + 0.2% turmeric flour + 0.1% probiotic; T4= Basal diet + 0.8% turmeric flour + 0.6% probiotic; and T5 = Basal diet + Bazitracin 0.01% antibiotic.

Variables

The research variables were the diameter of the inhibition zone of Salmonella and *Escherichia coli* bacteria (mm) using the Kirby-Bauer method, Histology Total Plate Count, and the number of bacterial colonies Total Plate Count (cfu/g) (Zada, 2021).

Data Analysis

Data were analyzed using analysis of variance (ANOVA). If there was a difference between the treatments, proceed with the Least Significant Difference (LSD).

Results and Discussion

In vitro inhibition test of the effect of adding turmeric extract (*Curcuma longa*) and probiotics to *Salmonella sp* and *Escherichia coli*

The results of the analysis of the diameter of the inhibition zone from the effect of extracts from the effect of adding turmeric extract and probiotics to Salmonella and *Escherichia coli*are presented in Table 1.

Table 1. The results of the antibacterial activity test of the combination of turmeric and probiotics on the growth of *Salmonella* spand *Escherichia coli*

Treatments	Average inhibition zone diameter (mm)	
	***Salmonella* sp**	***Escherichia coli***
C0 (Control 0%)	0^{a}	0^{a}
C1 (turmeric 98%+ aquadest 2%)	$1{,}98\pm0{,}30^{b}$	$2{,}85\pm0{,}29^{b}$
C2 (probiotic 98% + aquadest 2%)	$3{,}91\pm0{,}71^{c}$	$4{,}94\pm0{,}49^{c}$
C3 (turmeric 92% + probiotic 8%)	$3{,}92\pm1{,}35^{c}$	$5{,}00\pm1{,}36^{c}$
C4 (Bazitracin 92% + aquadest 8%)	$5{,}13\pm0{,}00^{c}$	$5{,}49\pm0{,}00^{c}$

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Description: The same superscript in the same column showed no significant difference ($p>0.05$). Different superscripts in the same column showed a very significant difference. ($p<0.01$).

Table 1 showed that the lowest average diameter of the Salmonella bacteria inhibition zone is at C0, and the highest on C4 is 5.13 mm. Besides Salmonella, Table 1 also shows that the average diameter of the inhibition zone of *Escherichia coli* bacteria in treatment C0 was 0 mm and the highest was C4 treatment 5.49mm.

Based on the results of the analysis of variance in Table 1, the average diameter of the inhibition zone of *Salmonella sp* and *Escherichia coli* bacteria had a very significant effect ($p<0.01$) on the treatment. The results of further LSD test on the inhibition zone diameter of turmeric and probiotics against *Salmonella sp* showed that T0 was significantly different from C1, but C2, C3, and C4 did not show significantly different results. Likewise, the same results for *Escherichia coli* showed that T0 was very significantly different from C1, while C2, C3, and C4 showed no significantly different results.

The effect of adding turmeric flour and probiotics to the amount of gut microflora of Mojosari ducks (TPC)

The results of the calculation of the total plate count (TPC) of bacterial colonies with the addition of turmeric and probiotics can be seen in *Table 1*.

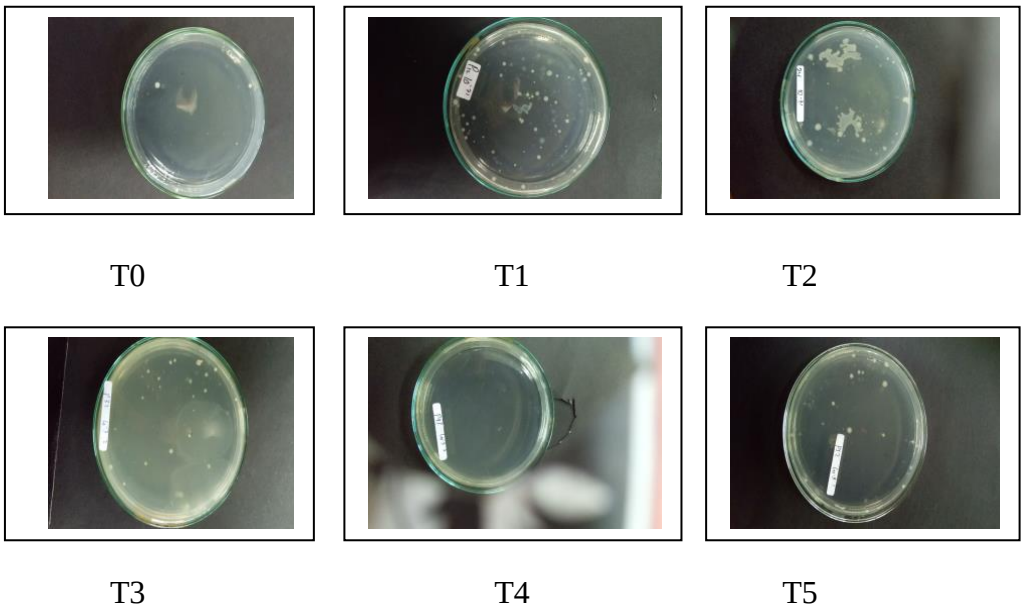

Figure 1. Total plate count of bacterial colonies with different treatments (T0) Basal diet + 0% control, (T1) Basal diet + Turmeric 0.2%, (T2) Basal diet + Turmeric 0.8%, (T3) Feed basal + Turmeric 0.2% + Probiotic 0.1%, (T4) Basal diet + Turmeric 0.8% + Probiotic 0.6%, (T5) Basal diet + Bazitracin 0.01%.

The average number of bacterial colonies Total Plate Count (TPC) cfu/gram in the intestines of Mojosari ducks is presented in Table 2.

Table 2. Average number of bacterial colonies Total Plate Count (cfu/gram)

Treatment	Average
T0 (Basal diet + 0% control)	$51{,}50\pm30{,}40^{a}$
T1 (Basal diet + Turmeric 0.2%)	$144{,}39\pm30{,}79^{b}$

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T2 (Basal diet + Turmeric 0.8%)	56,68±22,82[a,c,d]
T3 (Basal diet+ Turmeric 0,2% + Probiotic 0,1%)	85,10±17,05[c,d]
T4 (Basal diet + Turmeric 0,8% + Probiotic 0,6%)	95,92±33,28[c]
T5 (Basal diet + Bazitracin 0,01%)	48,33±22,50[a,d]

Description: The same superscript in the same column showed no significant difference ($p>0.05$).

The results of the analysis of variance on the average number of bacterial colonies Total Plate Count (TPC) (Table 2) showed a very significant effect ($p<0.01$) on the treatment where the lowest and the highest average number of bacterial colonies Total Plate Count (cfu/gram) were T1 (164.58±32.92 cfu/gram)and T5 (48,33±22,50), respectively.

Based on the results of Least Significant Difference (LSD) test, the average number of bacterial colonies, Total Plate Count (cfu/gram) in treatment T0 has a very significant difference of T1 ($p<0.01$). In contrast, the treatment T2, T3, T4, and T5 shows no significant difference ($p>0.05$). Treatment T1 showed a very significant difference ($p<0.01$) compared to the other treatments.

The use of antibiotics can lead to antibiotic resistance in livestock (Landers et al., 2012; Manyi-Loh et al., 2018; Najwan et al., 2019). Several studies have reported the existence of *Salmonella sp* resistance to antibiotics in ducks. According to Adzitey et al. (2012), *Salmonella sp* was found in duck farms, especially in the process of rearing and processing ducks. It was relatively high, with prevalence rates ranging from 0.0 to 39.0%. Loisa et al. (2016) showed that ducks reared on farms in Bogor Regency were polluted by *Salmonella sp.* This was proven by duck meat samples, which were positively contaminated by*Salmonella sp.*The prevalence of Salmonella incidence was 65.2% in duck farms in Pekin, South Korea.In comparison, the prevalence of *Salmonella serotypes* in ducks was 43.4%. (Cha et al., 2013). Thus,antibiotics have lost their effectiveness and tend to cause widespread microbial resistance. Therefore, to anticipate this issue, natural stuffsuch as turmeric isneeded to function as feed additives in poultry feed, especially ducks. Turmeric can be used as a natural growth promoter in poultry feed, because it is safe and has high pharmacologic benefits (Khan et al., 2012). Turmeric is also one of the herbal plants that can be used as an alternative to antibiotics in poultry feed (Dono, 2014; Walton, 1977). According to research, the addition of turmeric flour and probiotics in broiler feed can increase the number of small intestinal villi (Sjojan et al., 2020).

Table 1 shows that T3using a combination of 92% turmeric and 8% probioticshad moderate inhibition. The content of curcumin can produce the inhibition of bacteria as a probiotic. The previousstudy showed that curcumin couldinhibit various microorganisms such as Salmonella and *Escherichia coli* (Kim et al., 2005; Niamsa and Sittiwet, 2009). Davis and Stout (1971) stated that the diameter of the clear zone 10-20 mm had strong inhibition, the diameter of the clear zone of 5-10 mm had moderate inhibition, and the diameter of the clear zone <5 mm had weak inhibition. However, the diameter of the inhibition zone for turmeric and antibiotics was lower than the inhibition zone for antibiotics, which was 5.00 mm. This is because antibiotics come from microorganisms or substances produced by chemical synthesis. According to research, turmeric extract has the highest inhibition zone diameter than white turmeric, ginger, and encountering extracts, which is 5.64 mm with a minimum inhibitory level of 50% (Nurina et al., 2016).

The inhibition of Salmonella and *Escherichia coli* bacteria growth will increase along with the concentration of provided turmeric and probiotics because it contains more anti-bacterial properties. According to Tyagi et al. (2015) and Gul and Bakht (2015), turmeric rhizome

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powder on bacteria showed that turmeric rhizome extract caused the growth of *Escherichia coli*. Turmeric has the active compound curcumin, decreasing antibacterial activity (Adamczak et al., 2020; Teow et al., 2016). Its broad-spectrum properties are antibacterial, which is active against various types of gram-positive and gram-negative bacteria (Packiavathy et al., 2014), antiviral, and induces cell apoptosis (antitumor) and anti-cancer (Alok et al., 2017; Wilken et al., 2011). This shows that turmeric has a high potential as a substitute for antibiotics. Curcumin as a polyphenolic compound has an antimicrobial mechanism by inhibiting the thiolase enzyme (sulhydryl enzyme) until protein denaturation occurs (Fernandes et al., 2012). Polyphenols are also lipophilic compounds that can damage bacterial cell membranes (Bouarab-Chibane et al., 2019). Essential oil is a terpenoid compound whose antibacterial mechanism is thought to be through the process of destroying bacterial cell membranes (Chouhan et al., 2017). The research results of Janßen et al. (2001) showed that turmeric extract at a concentration of 50% (v/v) could inhibit the activity of *Escherichia coli* bacteria from increasing the production of non-pathogenic bacteria, as well as probiotics as antimicrobials that produce antimicrobial compounds that can inhibit the growth of pathogenic bacteria. Experiments on ducks using *Bacillus circulan*as a probiotic also did not detect the presence of *Salmonella sp*. in the intestines and eggs (Manin, 2003)

Treatment T1 in Table 2 shows a very high number of bacterial colonies compared to all treatments. This is possible because the amount of Lactic Acid Bacteria (LAB)in probiotics is too small (107 cfu/ml). LAB is a group of bacteria that have potential as probiotics because they have the ability to convert sugar into organic acids (lactic and acetic) which can function as probiotics (Bintsis, 2018; Naeem et al., 2012). Astuti et al. (2015) also reported that the minimum standard of probiotic lactic acid bacteria utilization is 106 cfu/ml. Providing probiotics at a 10-6 cells/ml concentration can increase livestock productivity (Shah, 2007). According to Zurmiati (2014), probiotics have a good effect on the growth of ducks and can increase lactobacillus byup to 8.3%. Feeding additives that do not contain probiotics can also increase the length and weight of the digestive tract in female Tegal ducks (Fandi et al., 2019).

Conclusion

The average diameter of the inhibition zone of Salmonella and Escherichia coli bacteria was highest in the treatment using 92% antibiotics + 8% distilled water. The addition of 0.8% turmeric flour in the feed gave the best results on the number of bacterial colonies Total Plate Count (TPC) in the intestines of Mojosari ducks.

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