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Acidifier Affects *In Vitro* Antimicrobial Effect and *In Vivo* pH and Intestinal Microflora in Laying Duck

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***Abstract*—** *The aims of this study were to evaluate in vitro antimicrobial effect of acidifier and in vivo pH and intestinal microflora in Laying Duck. Acidifier used were formic acid and fumaric acid. This research was divided into two stages. In first experiment, the method used was in vitro experimental method and using Completely Randomized Design (CRD) having 4 treatments and 5 replications. The treatments consisted of T0= aquadest+Bacitracin. T1= aquadest + 0.5% formic acid. T2= aquadest + 0.5% fumaric acid and T3= aquadest + 0.5% mixture of formic acid and fumaric acid at ratio of 50:50. Variables observed were diameter of the clear zone indicating inhibition activity Lactic Acid Bacteria (LAB), Escherichia coli, and Salmonella. At second experiment, in vivo experiment was carried out and using Completely Randomized Design (CRD). One hundred and fifty female ducks of 50 weeks old randomly distributed into 5 treatments and 5 replications. The treatments consisted of T0-= basal feed/control. T0+= basal feed with addition of Bacitracin 0.1%, T1= basal feed + 0.1% mixture of formic acid and fumaric acid, T2= basal feed + 0.2% mixture of formic acid and fumaric acid. T3= basal feed + 0.3% mixture of formic and fumaric acid at ratio of 50:50. The variables observed were population of intestinal pathogenic and non-pathogenic microflora. The result of first experiment showed that clear zone of inhibitory diameter significantly decreased (P>0.01) by any acidifier treatments for type of microbes. In the second experiment, the population of intestinal microflora significantly decreased pH and also significantly decreased population of LAB, Escherichia coli, and Salmonella.*

***Keywords*—** *Acidifier, inhibition zone diameter, intestinal microflora, formic acid, fumaric acid.*

I. INTRODUCTION

Antibiotic Growth Promotor (AGP) commonly used to increase the production, but recently, the prohibition of antibiotic added into feed mixture either in broiler or layer due health concern. Since antibiotic was banned by government to be used as growth promoter, many farmers are suffered because of their production significantly dropped. Therefore, current researches are mostly focused on finding the alternative replacer for antibiotic.

Acidifier is one of the replacer for antibiotic that can be added into feed mixture for performance improvement, like benzoic acid, fumaric acid, formic acid, etc. There are two kind of acidifiers based on its form, solid and liquid. Solid form has an advantage because it is protected when it passed through the gut. On it application, acidifiers are able to escalate the intestinal gut mucosa and its morphology of birds, organic acid can also improve immunity, reducing pathogen activity and balancing the population of bacteria in gut. These kind of acids can make ideal environment for lactic acid bacteria to grow and help to inhibit pathogenic microflora to develop. In the previous research, it was reported that Salmonella growth can be minimized by lowering pH in gastrointestinal intact by using acidifier [1]. This research aimed to examine *in vitro* antimicrobial effect and *in vivo* evaluation of intenstinal pH and microbial population in laying duck.

II. MATERIAL AND METHOD

This experiment was held in two stages, *in vitro* in first experiment, then followed into *in vivo* in second experiment.

A. The First experiment

In first experiment, *in vitro* method was applied by using Completely Randomized Design (CRD) having 4 treatment and 5 replication. The treatments which were used in this experiment consisted of:

T0 : aquadest + Bacitracin.
T1 : aquadest + 0.5% formic acid.
T2 : aquadest + 0.5% fumaric acid.
T3 : aquadest + 0.5% mixture of formic and fumaric acid.

The variables observed in this experiment were diameter of inhibition zone observed for 3 different microflora, namely Lactic Acid Bacteria (LAB), *Salmonella*, and *Escherichia coli*. The equipments used were Petri dish, test tube, erlenmeyer, incubator, Ohaus balance, micropippet 1 ml, autoclave, waterbath and magnetic stirrer.

Measurement of diameter of inhibitory zone was by using the method of Pradikdo, *et al.* (2019) utilizing caliper to measure 2 directions: horizontal and vertical on clear zone circle. The diameter of inhibition zone was measured by different between clear zone and original hole diameter.

B. The Second Experiment

Birds and Dietary Treatments

One hundred and fifty 50 weeks old of Mojosari female laying ducks. Each experimental unit had an area of 0.4 m^2. The lighting used was 2 ten watt of lamp provided between 17.00 p.m. to 5.00 a.m. Feed was given restricted as much as 160 g/bird. Water was given *ad libitum*. The formula and nutrient content of basal diets were described in this following Table:

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TABLE I. Formula of basal feed

Raw Materials	Proportion (%)
Yellow corn	48.00
Soy bean meal	21.00
Rice bran	12.20
Meat bone meal	8.00
Soybean oil	2.00
Grit	5.00
DL-methionine	0.20
Mineral premix	2.00
Vitamin premix	0.50
Salt	0.10
Total	100.00

TABLE II. Nutrient content of basal feed

Nutrient	Proportion (%)
Crude protein (%)	19.34
ME (kcal/kg)	2.800
Fiber (%)	4.5
Fat (%)	4
Calcium	3
Phosphor	0.5
Lysine	1.05
Methionine	0.5

In second experiment, *in vivo* method was applied. The experimental design was Completely Randomized Design (CRD) consisting of 5 treatments and 5 replication with 6 ducks for each replication. The 150 laying ducks were randomly distributed into treatments and replications. The treatments given were:

T0- : basal feed/control
T0+ : basal feed with addition of Bacitracin antibiotic 0.1%
T1 : basal feed + 0.1% mixture of formic acid and fumaric acid
T2 : basal feed + 0.2% mixture of formic acid and fumaric acid
T3 : basal feed + 0.3% mixture of formic and fumaric acid at ratio of 1:1

The variables observed in this experiment were intestinal microflora and the pH of intestinal content. Ileum part of gut was taken, cut in 2 cm, and put into a pot and labelled for each treatments. pH value measured [3] by taking 1g of digesta in ileal then added with aquadest up to 10 ml. the mixture then centrifuged at 3000 rpm for 5-10 minutes. The supernatant was separated and put into beaker glass for pH measurement. Total population of *Escherichia coli*, *Salmonella* sp. and LAB were calculated by using Total Plate Count (TPC) [4].

C. Statistical Analysis

Data obtained from this research analyzed with one way analysis of variance (ANOVA) then continued with Duncan's Multiple Range Test (DMRT) if there were significant differences.

III. Result and Discussion

A. Diameter of Inhibition Zone

The result of diameter of inhibition zone due to treatment of acidifiers is showed in Table III.

TABLE III. Effect of acidifier on inhibition zone.

Treatments	*Escherichia coli* (mm)	*Salmonella sp.* (mm)	*Lactobacillus* (mm)
T0	4.04 ± 0.04^{d}	4.10 ± 0.06^{d}	4.33 ± 0.14^{d}
T1	2.20 ± 0.06^{b}	2.53 ± 0.05^{b}	2.32 ± 0.10^{b}
T2	2.01 ± 0.08^{a}	2.35 ± 0.03^{a}	2.16 ± 0.03^{a}
T3	3.06 ± 0.03^{c}	3.46 ± 0.17^{c}	3.31 ± 0.04^{c}

Notes: The different superscripts in the same row showed highly significant differences ($p<0.01$)

The result showed that the mixture of formic acid and fumaric acid could exert similar effectiveness of bacitracin antibiotic in term of ability to suppress growth of bacteria, which was T3 3.06±0.03 mm compared to T0 4.04±0.04 mm of antibiotic to inhibit growth of *Escherichia coli* (Table III), the wider the clear zone indicated that the less growth of bacteria. In fact, the result showed that using single acidifier (for T1 and T2) showed less effective than combination of acidifier or the use of antibiotic. Therefore, ability of acidifier mixture was still significantly lower than antibiotic. Previous report indicated that the use of organic acid can inhibit the growth of *Escherichia coli* at pH 4 [5]. Similiar result showed for the effectiveness of the mixture of formic acid and fumaric acid in term of inhibiting the growth of *Salmonella*. *Salmonella* will grow at optimum range of pH 7-7.5. Outside the pH range for optimum growth, cells may become inactive [6] [7]. Lactic acid bacteria is an end product of Lactobacillus fermentation that produces lactic acid. Lactic acid bacteria have greater difficulty to grow in this environment as they are less well adapted to the high sugar concentrations (>210 g/L) and low pH of the must (3.0–3.3) [8] [9].

B. Effect of Acidifiers on Ileal Characteristic

The result of acidifier addition in feed to the ileal microbes and pH is showed in Table IV:

TABLE IV. Effect of acidifier on ileal microbes and pH.

Treatments	*Escherichia coli* (CFU/g)	*Salmonella sp.* (CFU/g)	*Lactobacillus* (CFU/g)	pH
T0-	5.38 ± 0.17^{e}	6.23 ± 0.11^{e}	4.34 ± 0.06^{e}	6.72 ± 0.06^{c}
T0+	2.45 ± 0.16^{a}	2.94 ± 0.15^{a}	1.46 ± 0.40^{a}	6.6 ± 0.40^{c}
T1	3.48 ± 0.16^{b}	3.89 ± 0.05^{b}	2.49 ± 0.15^{b}	6.58 ± 0.15^{c}
T2	4.12 ± 0.20^{c}	4.7 ± 0.13^{c}	3.23 ± 0.10^{c}	6.3 ± 0.10^{b}
T3	4.67 ± 0.36^{d}	5.37 ± 0.24^{d}	3.98 ± 0.13^{d}	5.9 ± 0.13^{a}

Notes: The different superscripts in the same row showed highly significant differences ($p<0.01$)

Table IV showed the effect of acidifier on average total population of pathogenic bacteria (*Escherichia coli* and *Salmonella*) and non-pathogenic bacteria (*Lactobacillus*). Acidifier given was expected to suppress the pathogenic bacteria growth and also increasing the population of lactic acid bacteria. Highest total of lactic acid bacteria as shown in

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Table IV was T0- with the average 4.34±0.06 CFU/g, followed by T3 which has an average 3.98±0.13 CFU/g, while the lowest total was T0+ 1.46±0.40 CFU/g. The analysis of variance result showed that the acidifier as feed additive gave a highly significant effect ($p<0.01$) to the population of non-pathogenic bacteria.

The highest population of *Escherichia coli* showed at Table IV. was T0- 5.38±0.17 CFU/g while the lowest amount at T0+ 2.94±0.15 CFU/g. From the table shown, basal feed with no addition of either antibiotic or acidifier, had the highest population of *Salmonella* sp of T0- 6.23±0.11 CFU/g, while the lowest was T0+ 2.45±0.16 CFU/g. the basal feed with no addition of either antibiotic or acidifier, had the highest amount of bacteria, regardless the pathogenic and non-pathogenic bacteria. Meanwhile, the use of antibiotic mostly kills the microorganism in gut, both pathogenic and non-pathogenic microbe. Though less effective than antibiotic, T1 showed a good ability to reduced pathogenic bacteria with mild reduction in *Lactobacillus*.

Based on Table IV. result in intestinal gut pH showed highly significant result ($p<0.01$) when acidifier was given as feed additive to laying duck. T3 showed the lowest pH average of 5.9±0.13 while the highest average pH showed at T0-. However, T1 showed no different pH as compared to T0- and T0+. Level of pH in specific areas in gastrointestinal tract is important in term to establish a specific microbial population and can also affect the digestibility and absorptive ability of gut for most nutrient. Most of pathogens grow in pH around 7. In contrast, beneficial microorganisms live in more acidic pH (5.8-6.2) [10]. Organic acid given to poultry is more likely has a direct role on the GIT bacteria population, reducing the level of pathogens and controlling the population of certain types of bacteria which compete for nutrients in the gut [11]. Lactic acid bacteria (LAB) living symbiotically in the digestive tract of birds and found to enhance the immunity of birds to pathogenic bacteria [8]. Commercial mixture of organic acid that consisted of fumaric acid, calcium format, calcium propionate, potassium formate and hydrogenated vegetable oil, significantly reduced intestinal *Escherichia coli* and *Salmonella* compared to Enramycin antibiotic that only reduced *Escherichia coli* but had no effect on *Salmonella* counts [12].

IV. Conclusion

Based on the results, the mixture of formic acid and fumaric acid 0.1% showed the best result in term of competing with bacitracin acidifier based on diameter of inhibition zone. Meanwhile, the mixture of formic acid and fumaric acid 0.3% showed best result in term to suppress the pathogenic bacteria (*Escherichia coli* and *Salmonella*).

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