The effectiveness of cassava waste and skim milk as a filler of phytogenic and probiotic blends to inhibit the pathogenic bacteria and aflatoxin

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Abstract. This research aims to determine the effectiveness of cassava waste and skim milk as a filler of phytogenic and probiotic blends to inhibit phtytogenic bacteria and aflatoxin. The phytogenics and probiotics blends were used in the yellow type with the composition of Galangal *(Kaempferia rhizome)*, Temulawak *(Curcuma xanthorrihiza roxb)*, Red Ginger *(Zingiber officinale)*, Turmeric *(Curcuma domestica val)*, Actinomycetes, lactic acid bacteria, photosynthetic bacteria, tempeh yeast, and fermentation fungi *(Aspergillus)*; furthermore, the green type consists of Sambiloto (*Andrographis paniculata*), Betel *(Piper betle)*, Moringa *(Moringa Oliefera)*, Papaya *(Carica papaya)*, actinomycetes, lactic acid bacteria, photosynthetic bacteria, tempe yeast, and fermentation fungi *(Aspergillus)*. Each pytobiotic and probiotic in yellow and green types were filled with cassava waste and skim milk, then dried in an oven at 50 °C for 24 hours. The ratio between the combination of phytogenics and probiotics with the filler is 1:1. The inhibition was divided into four types, consisting of positive control, negative control, cassava waste, and skim milk. The inhibitory pathogenic bacteria and fungi used the Well Method. The data were analyzed using a complete randomized design. If there were a significantly different result, then the analysis of the Duncan Multiple Range Test (DMRT) would be continued. The results showed that control positive in the yellow and green type has the higher inhibitory pathogenic on salmonella, escherichia coli, and aflatoxin. However, the filler casava waste has higher inhibitory salmonella, escherichia coli, and aflatoxin compared to skim milk. The conclusion of this research showed that cassava waste is effective as a filler for phytogenic and probiotics as an inhibitory pathogenic bacteria and aflatoxin.

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1 Introduction

Salmonella bacteria have a significant impact on poultry production and cause systemic disease in broilers. *Salmonella* contamination of poultry products, such as eggs, is a major concern for food safety and human health. *Salmonella* can pass through feces, contaminating egg surfaces and posing a risk to consumers. *Salmonella* has a wide host range, can colonize the gastrointestinal tract of poultry, and transfer antibiotic resistance genes to other bacteria, thus contributing to the prevalence of drug-resistant microbes in commercial poultry. Therefore, it is important to find prophylactic options that can reduce the number of bacteria and increase poultry production. *Escherichia Coli* is a poultry pathogen that can cause systemic disease in both broilers and laying hens, causing high mortality rates. *Escherichia Coli* bacteria often contain many virulence factors and antimicrobial resistance, making them difficult to control. These bacteria can interact with heterophiles and macrophages of broilers, thus causing severe infections. It is important to find effective measures to reduce the colonization of *Escherichia Coli* and improve poultry health [21]. The poison produced by fungi, namely aflatoxin, can also be harmful to poultry production. This can inhibit the formation of liver proteins and lipid metabolism in the liver, causing a swollen and fatty liver. Aflatoxin can also lower cholesterol levels in broiler's serum and affect the role of hepatocytes. In addition, aflatoxin can contaminate feed and reduce its quality, thus negatively impacting poultry performance [1].

Probiotics can be used as an alternative to prevent the growth of *Salmonella* and *Escherichia Coli* bacteria. Such ingredients can increase the immunity of broilers and improve the vaccination response, thereby increasing protection against bacterial infections. Probiotics have been shown to stimulate the production of natural antibodies in broilers, which can help fight *Escherichia Coli* and *Salmonella* In addition, probiotics have the potential to reduce the number of bacteria and increase overall poultry production. It is important to continue to research and develop these strategies in order to effectively control *Escherichia Coli* and *Salmonella* in poultry and ensure optimal poultry health and food safety [21]. Phytogenic are natural and residue-free compounds derived from plants that have been used as feed additives for livestock production. They include various bioactive components or substances of plant origin such as terpenoids, alkaloids, glycosides, and phenolics. Phytogenic also has antimicrobial properties, fighting intestinal pathogenic microorganisms while maintaining a population of beneficial microflora. In addition, phytogenic has an immunostimulant effect on humoral and cellular immunity and has antioxidant properties. They are used as growth-promoting feed additives, antimicrobial agents, and immunostimulants in the poultry industry [7].

A combination of phytogenic and probiotic products. Type H phytogenic and probiotic (Green) with the composition of Sambiloto *(Andrographis paniculata),* Betel *(Piper betle)*, Moringa *(Moringa Oliefera)*, Papaya *(Carica papaya)*, actinomycetes, lactic acid bacteria, photosynthetic bacteria, tempeh, and fermented fungi *(Aspergilus)* and type K phytogenic and probiotic (Yellow) with the composition of Kencur *(Kaempferia rhizome)*, Temulawak *(Curcuma xanthorrihiza roxb)*, Red Ginger *(Zingiber officinale)*, Turmeric *(Curcuma domestica val)*, Actinomycetes, lactic acid bacteria, photosynthetic bacteria, tempeh yeast, and fermentation fungi *(Aspergilus)*. The bioactive content of phytogenic and probiotics contains antibiotic and antimicrobial compounds that are expected to inhibit the growth of *Salmonella*, *Escherichia Coli*, and aflatoxin fungi in the form of mash. The purpose of this study was to determine the best filler that will be combined with probiotics and phytogenic to inhibit *Salmonella*, *Escherichia Coli*, and aflatoxin fungi.

2 Material and Method

The method is carried out through 2 stages, namely, making a mixture of feed additives with fillers two kinds of filler, and the second is the inhibitory power test. The mixture was carried out in the Laboratory of Animal Feed Nutrition and the inhibitory pathogenic in the Epidemiology Laboratory of the Faculty of Animal Science, Universitas Brawijaya, Malang.

2.1 Mixing of Feed Additive with Fillers

The production of mash-shaped phytogenic and probiotics has 2 types of fillers used, namely cassava waste and skim milk. Mixing phytogenic and probiotic with each type of filler with the ratio of 1: 1 which is then homogenized by stirring. Then the mixture of phytogenic and probiotic mixture dough with each filler is applied thinly to the baking sheet. After spreading evenly, the dough is put in a 50°C oven for 24 hours. After that the dough will dry completely and take the mongering dough from the pan and puree it with a mixer.

2.2 Inhibition Test

2.2.1 Media Production

MHA (Mueller Hinton Agar) media is made by weighing 15.2 grams then dissolved into 400 mL aquades with a magnetic stirer by heating to boiling. Then the media is sterilized using an autoclave with heating to 121°C. Sterilized Mueller Hinton Agar media is poured into sterile petri dishes and allowed to stand at room temperature until solidified.

2.2.2 Bacteria Culture

SA (Sodium Agar) media weighed 0.4 grams then dissolved into 20 mL of aquades homogenized with a magnetic stirer, heat until boiling. Prepare 9 mL of peptone then sterilize both using an autoclave until the temperature is 121°C. Bacteria are taken from pigeon feces which will then be spread to SA (Sodium Agar) media isolated using selective media. Take 1 gram of pigeon feces, dissolve it with 1 mL of sterile aquades then fortex until homogeneous. Take 1 mL of feces using a reconstituted micropipette and place it in a sterile petri dish. Then pour sterile NA media into a petri dish containing feces and homogenize it. Let stand until the substrate hardens at room temperature. After hardening, put in the incubator with a temperature of 37 °C for 24 hours. So that colonies are formed.

2.2.3 Bacteria Isolation

Each bacterial isolation medium weighed, namely SSA (Salmonella Shigela Agar) for *Salmonella* 1.26 grams, EMBA (Eosin Methylen Blue Agar) for *Escherichia Coli* bacteria 0.75 grams, and MEA (Malt Agar Extract) for aflatoxin 0.96 grams each dissolved into aquades 20 mL and homogenized at once by heating to boiling using a magnetic stirer. Then sterilized using an autoclave to a temperature of 121 ^{0}C. Then pour into a sterile petri dish and let it stand until it hardens. Bacteria emerging from NA media were taken using swabs then stricked into each selective media. Put in the incubator with a temperature of 37°C for 24 hours.

2.2.4 Inhibition Test

MHA media weighed 22.8 grams dissolved into 600 mL of homogenized aquades and heated to boiling using a magnetic stirrer. Take a small amount of each colony of *Salmonella*, *Escherichia Coli*, and Aflatoxin bacteria using a swab. Then tricked into a cup containing MHA media. Form a wells by perforating the media with a kock borer then fill the wells hole with a phytogenic and probiotic sample of 0.1 mL. Then put in an incubator with a temperature of 37°C for 24 hours. Then measure the clear zone around the wells using a caliper.

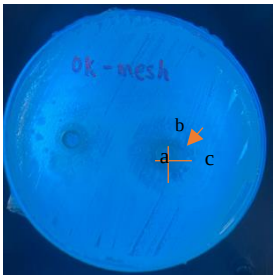

Figure 1. Measurement Inhibition Zone

Information :
a : horizontal diameter
b : vertical diameter
c : diameter of wells
The diameter of the inhibitory zone can be calculated using the following formula:

$$Inhibitory\ zone = \left(\frac{a+b}{2}\right) - c \quad (1)$$

2.3 Statistical Analyse

The experiment was conducted in duplicate and the values were written in the average ± standard deviations tabulated in Microsoft Excel. Then continued statistical analysis was carried out using a one-way-analysis of variance (ANOVA) (SPSS version 27). Then continued with Duncan's analysis (LSD) on a statistically significantly different treatment ($P<0.05$).

3 Results and Discussion

Testing the inhibitory zone of *Salmonella*, *Escherichia Coli*, and aflatoxin fungi on a combination of phytogenic with probiotics using the wells method. There are 2 types of phytogenic and probiotic with different phytogenicsotic content, namely yellow phytogenic and probiotic and green phytogenic and probiotic. Phytogenic and probiotic in the form of mash using cassava waste filler and skim milk. Table 1 has presented the results of inhibitory power tests of both types of phytogenic and probiotic and two types of fillers.

3.1 The Effect of Phytogenic and Probiotic Yellow Type on The Growth Inhibition of Salmonella sp, Escherichia Coli, and Aflatoxin

Testing the inhibitory zone of *Salmonella*, *Escherichia Coli*, and aflatoxin on a combination of phytogenic with probiotics using the wells method. There are 2 types of phytogenic and probiotic with different phytogenicsotic content, namely phytogenic and probiotic yellow type and phytogenic and probiotic green type. Phytogenic and probiotic in the form of mash using cassava waste filler and skim milk. Table 1 has presented the results of inhibitory power tests of both types of phytogenic and probiotic and two types of fillers.

Table 1. Result of Inhibition Test *Salmonella sp.*, *Escherichia coli,* and Aflatoxin

Bacteria	Yellow Type				Green Type			
	Control +	Control -	Cassava Waste Filler	Skim Milk Filler	Control +	Control -	Cassava Waste Filler	Skim Milk Filler
Salmonella	35.25 ± 0.78	0.00 ± 0.00	5.76 ± 0.23	2.86 ± 0.09	34.85 ± 0.21	0.00 ± 0.00	2.86 ± 0.09	0.75 ± 0.07
E. coli	34.07 ± 1.15	0.00 ± 0.00	3.83 ± 0.81	1.64 ± 0.58	33.88 ± 0.88	0.00 ± 0.00	7.98 ± 0.39	0.86 ± 0.41
Aflatoxin	33.07 ± 1,12	0.00 ± 0.00	0.50 ± 0,14	0.26 ± 0.02	33.02 ± 1.20	0.00 ± 0.00	0.50 ± 0.35	0.13 ± 0.04
Average	33.13 ± 0.91	0.00 ± 0.00	3.36 ± 2.66	1.59 ± 1.30	33.92 ± 0.92	0.00 ± 0.00	3.78 ± 3.82	0.58 ± 0.39

Phytogenic and probiotic yellow type found the diameter of the inhibitory zone of the highest value was produced in positive control (33.25 ± 0.91), onggok filler (3.36 ± 2.66), skimmed milk filler (1.59 ± 1.30), and negative control (0.00 ± 0.00). The positive control gave the highest inhibitory zone diameter value due to the administration of antibiotics in the form of ciprofloxacin which can significantly inhibit the growth of *Salmonella sp* bacteria, *Escherichia Coli* bacteria, and aflatoxins. Cassava waste filler given a lower value than positive control but higher than skim milk filler and negative control. This is due to the huge starch content ranging > 65.90% which is the main form of complex carbohydrates in the product [6]. Starch can also be converted into glucose which can be a source of energy for probiotic bacteria present in bioherbs such as actiomycetes, LAB (Lactic Acid Bacteria), and fermented fungi (Aspergilus). While the value of the inhibitory zone in skim milk filler is lower than the positive control and cassava waste filler but higher than the negative control, this is due to the fact that skim milk does not contain significant amounts of glucose [21]. This also causes the value of the inhibitory zone in skim milk fillers to be lower when compared to onggok fillers which contain a lot of starch, an energy source of several probiotics.

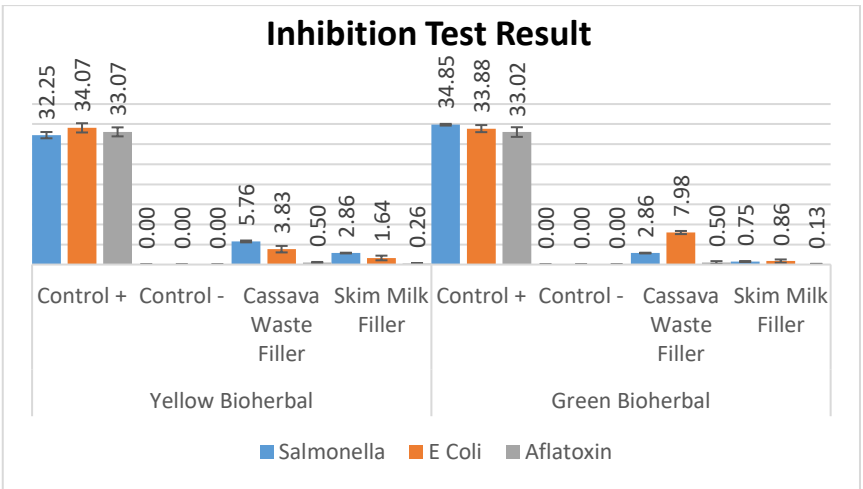

Fig. 2. Inhibition Test Result Diagram

The formation of inhibitory zones in yellow phytogenic and probiotic types against *Salmonella sp.* and *Escherichia Coli* is caused by several bioactive compounds it contains, namely the *Zingiber officinale* plant, namely ginger which can inhibit bacterial growth, including compounds such as Allicin, Allistatin, Ajoene, Gingerol, Gingerdiol, and Gingerdione. These compounds have therapeutic properties and play an important role in metabolism and antibiotic activity [12]. *Curcuma xanthorrhiza Roxb* contains curcumin which effectively inhibits bacterial growth and biofilm formation [27]. The antibacterial effect of *Curcuma domestica* is provided quickly financial and F. Discover ne domestica may be due to cell wall differences from Gram-positive and Gram-negative bacteria, allowing easier infiltration of hydrophobic compounds and increasing inhibitory effects on bacteria [17]. The purity of curcumin in extracted *Curcuma domestica* was found to be quite high compared to pure curcumin samples. The celery extraction with a concentration showed a higher inhibitory power compared to the use of antibiotics against *Salmonella* and *E Coli* bacteria [31]. The maximum weight percentage of curcumin is reported at 5% [18]. The probiotics in bioherbs also help inhibit *Salmonella sp*. bacteria. and *E Coli*. Actinomycetes are considered a source of bioactive compounds and are capable of producing various classes of antibiotics with diverse chemical structures and mechanisms of action. Actinomycetes produce different classes of antibiotics, including aminoglycosides, peptides, ansamycins, B-lactams, tetracyclines, macrolides, lincosamides, epoxides, and aminocoumarins. These antibiotics work on specific prokaryotic targets without damaging the eukaryotic system [8]. Actinomycetes contain about 75% of antibiotics, mainly antibacterial, produced by. This antibiotic has shown high effectiveness against the inhibition of Gram positive and Gram negative bacteria [16]. Lactic acid bacteria have several mechanisms that contribute to their ability to inhibit bacterial growth. One of the mechanisms is the production of antimicrobial compounds, such as organic acids (lactic, citric, acetic, fumaric, and malic acid), hydrogen peroxide , CO2, diacetyl, ethanol, reuterin, acetaldehyde, acetoin, ammonia, bacteriocin, bacteriocin, bacteriocin-like inhibition. In addition, lactic acid bacteria compete with pathogenic microorganisms for nutrition, which further contributes to antimicrobial effects [3]. Rhizopus oryzae can produce organic acids such as lactic acid, which has been shown to have antibacterial effects.

The formation of inhibitory zones in the yellow type phytogenic and probiotic against aflatoxin is caused by antifungal compounds in *Curcuma xanthorrhiza* called xanthorrhizol. It has been reported to reduce the germination of conidia of various fungi, including A. flavus, A. fumigatus, A. niger, F. oxysporum, R. oryzae, and T. mentagrophytes. Curcumin and demothoxycurcumin compounds inhibit fungal growth by damaging various cellular processes, such as damaging cell membranes, inhibiting cell wall synthesis, and disrupting fungal enzymes and proteins [4]. Actinomycetes isolated from hypersaline soil have shown antifungal activity against various fungi. Overall, actinomycetes are considered a good source of antibacterial and antifungal compounds. Antimicrobial compounds produced by actinomycetes can inhibit the growth and development of fungi by targeting specific cellular processes or structures [26]. In LAB, especially Lactobacillus plantarum, it has been shown to reduce aflatoxin production. In one study, Lactobacillus plantarum showed antifungal and antimycotoxigenic activity against *Aspergillus flavus* and *Fusarium verticillioides*, which are aflatoxin-producing fungi [22]. Yeast helps control aflatoxin through its binding capacity. The yeast cell wall contains glucomannan compounds that can bind to aflatoxin thereby preventing the harmful effects of aflatoxin [2]. Antiaflatoxin in *Aspergillus* involves evaluating the antifungal properties of various phytochemicals, such as ascorbic acid, gallic acid, caffeine, and quercetin. This phytochemical has been tested against two strains of *Aspergillus*, namely *Aspergillus flavus* and *Aspergillus parasiticus*. The results showed that the four phytochemicals succeeded in reducing the growth and activity of the two test

mushrooms. Quercetin showed the highest inhibitory effect on both strains of *Aspergillus* [28].

3.2 The Effect of Phytogenic and Probiotic Green Type on The Growth Inhibition of Salmonella sp, Escherichia Coli, and Aflatoxin

Green type phytogenic and probiotic found the diameter of the inhibitory zone from the highest values produced in positive control (33.92 ± 0.91), onggok filler (3.78 ± 3.82), skimmed milk filler (0.58 ± 0.39), and negative control (0.00 ± 0.00). The positive control gave the highest inhibitory zone diameter value due to the administration of antibiotics in the form of ciprofloxacin which can significantly inhibit the growth of *Salmonella sp* bacteria, *Escherichia Coli* bacteria, and aflatoxins. Cassava waste filler given a lower value than positive control but higher than skim milk filler and negative control. This is due to the large amount of starch content in it which certainly affects probiotics, namely increasing their antioxidant content when put into biocomposite films and making energy sources for probiotic metabolism [1]. While the value of the inhibitory zone in skim milk filler is lower than the positive control and cassava waste filler but higher than the negative control, this is due to the fact that skim milk does not contain significant amounts of glucose [21]. This also causes the value of the inhibitory zone in skim milk fillers to be lower when compared to onggok fillers which contain a lot of starch, an energy source of several probiotics.

The formation of inhibitory zones in green phytogenic and probiotic types against *Salmonella sp.* and *Escherichia Coli* due to the content of phytogenic and probiotic bioactive compounds, such as in *Andrographis paniculate* there are antimicrobial compounds that can inhibit bacteria through various mechanisms. For example, andrographolide compounds can affect the Quorum Sensing System (QSS), the communication system between bacteria, thereby reducing biofilm production and inhibiting virulence factors. It can also restore antibiotic sensitivity in P. aeruginosa by reducing efflux pump expression. Other compounds in *Andrographis paniculata* have been shown to damage bacterial cell membranes, inhibit bacterial enzymes, and impair bacterial DNA replication and transcription [13]. Antimicrobial compounds found in *Piper betle*, such as hydroxychavicol, eugenol, chavibetol, and allylpyrocatechol, can inhibit bacteria through various mechanisms. For example, hydroxychavicol has been shown to disrupt bacterial cell membranes, leading to cell death. Eugenol has been found to inhibit bacterial cell wall synthesis and interfere with bacterial DNA replication. Chavibetol has been shown to inhibit the growth of some strains of bacteria, including Staphylococcus aureus, by disrupting bacterial cell membranes. Allylpyrocatechol has been found to inhibit the growth of several strains of bacteria, including Escherichia and Pseudomonas aeruginosa, by interfering with bacterial DNA replication and transcription. Overall, the antimicrobial activity of *Piper betle* is likely due to a combination of these mechanisms, as well as other factors such as concentration and mode of delivery of the compound [11]. In addition, Rhizopus oryzae may produce enzymes that may have antimicrobial properties that may inhibit pathogenic bacteria [19]. *Aspergillus* acts as an antibacterial agent through the production of secondary metabolites called antimicrobial compounds. The compound is produced as a fungal defense mechanism to inhibit bacterial growth in its environment [23]. Actinomycetes are known to be a rich source of natural products, including antibiotics. Mycelial bacteria produce most of the antibiotics that help in inhibiting the growth of *Salmonella sp*. and *Escherichia Coli* [30]. The antibacterial properties of their potential LAB (Lactic Acid Bacteria), which can be attributed to the production of organic acids, hydrogen peroxide, and bacteriokine. These substances help inhibit the growth of harmful bacteria and contribute to the preservation of food. In addition, lactic acid bacteria have been investigated for their potential use as probiotics, which are beneficial bacteria that can improve gut health and boost the immune system [10].

The formation of an inhibitory zone in the green type phytogenic and probiotic against aflatoxin is due to the herbal plant *Andrographis paniculata* containing several antifungal compounds. One of its main active compounds is andrographolide, which has been extensively studied for its antifungal properties by damaging fungal cell membranes leading to leakage of cell contents and cell death. It can also inhibit the growth and reproduction of fungi by interfering with the metabolic processes of fungi. In addition, andrographolide has been found to modulate the immune system and increase the body's natural defenses against fungal infections [13]. Antifungal activity in actinomycetes that produces compounds that can inhibit the growth and reproduction of fungi, thereby preventing or treating fungal infections. The specific compound mentioned in the document is Caerulomycin A [5]. The production of organic acids and other metabolites in LAB (Lactic Acid Bacteria) creates an unfavorable environment for fungal growth. In addition, lactic acid bacteria can also produce antifungal compounds called bacteriocins, which can inhibit the growth of certain fungi [10]. Disruption of cell membranes in the antifungal compound *Aspergillus* leads to leakage of cell contents and eventually cell death. Another class of antifungal agents, polyenes such as amphotericin B, bind to ergosterol in fungal cell membranes, forming pores that disrupt membrane integrity and cause cell death. Echinocandins, such as caspofungin and micafungin, inhibit the synthesis of beta-glucan, a key component of fungal cell walls, leading to weakening of cell walls and cell death. These are just a few examples of mechanisms by which antifungal agents target and kill *Aspergillus* fungi [29].

4 Conclusion

The conclusion that can be drawn from this study is the best filler that can help in inhibiting the growth of *Salmonella, Escherichia Coli*, and aflatoxin from phytogenic and probiotic type yellow namely cassava waste and phytogenic and green probiotic type which is also cassava waste.

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