Journal of Veterinary Science & Medical Diagnosis

Research Article A SCITECHNOL JOURNAL

In Vitro Study of *Curcuma*, Honey, and Probiotics Combination as Candidates for Feed Additives to Replace Growth Promoter Antibiotics (AGP)

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Received date: 07 December, 2022, Manuscript No. JVSMD-21-49191;

Editor assigned date: 10 December, 2022, PreQC No. JVSMD-21-49191 (PQ);

Reviewed date: 27 December, 2022, QC No. JVSMD-21-49191;

Revised date: 07 February 2022, Manuscript No. JVSMD-21-49191 (R);

Published date: 01 April 2022, DOI: 10.4172/2325-9590.11.3.016

Abstract

Growth Promoter Antibiotics are used to prevent disease and promote growth and production in poultry. Repeated administration of feed can have a micro-organic resistance effect, accumulation of antibiotic residues in animal and environmental products and imbalance of normal micro-flora in the intestine. The antibacterial and carbohydrate content of some natural ingredients can be potential as a replacement candidate for AGP. This study aims to determine the role of a combination of curcuma, honey, and probiotics (*Bacillus subtilis* and *Lactobacillus acidophilus*) as AGP candiate *in vitro*. The antibacterial activity of the combination of curcuma and honey against pathogens (*E. coli*) and their use against probiotics was tested by disk diffusion method, while the calculation of optical density values to determine the minimum inhibitory concentration and minimum bactericidal concentration was carried out on *E. coli*. The inhibition ability of probiotics against pathogens is also done by the disk diffusion method. The disk diffusion test results showed the best combination of 25% *curucuma aquades* extract+100% Lombok honey with inhibition zone diameter (8.53 ± 0.03). Optical density values indicate this combination is able to inhibit and kill *E. coli* (DO 0.00 ± 0.002) and supports the growth of *B. subtilis* (DO 0.18 ± 0.002) and *L. acidophilus* (DO 0.25 ± 0.005) significantly better than positive control. MIC value of *curcuma aquades* extract and honey combination against E. coli is curcuma aquades extract 3.13%+Lombok honey 25%, and MBC value is *curcuma aquades* extract 6.25%+Lombok honey 25%. The combination of *B. subtilis* and *L. acidophilus* probiotics showed the largest inhibitory zone diameter against *E. coli* pathogen (7.30 ± 0.02 mm) compared to individual colonies. The combination of curcuma and honey, in addition to inhibiting also able to kill pathogens and support the growth of probiotics, so this formula can be used as one of the replacement candidates for AGP.

Keywords: Antibiotic growth promoter; *E. coli*; Honey; Probiotics; *Curcuma*

Introduction

Antibiotics are used with the aim of increasing growth and production as well as preventing disease in poultry. These positive effects can change function when antibiotics are not in accordance with the recommendations and prescribed doses, so that they can have negative effects such as resistance to microorganisms, reduced use of good bacteria in the intestines, and antibiotic residues in animal products and the environment [1]. The Food and Agriculture Organization stated that the incidence of antibiotic resistance in both humans and animals is a major global problem and has now been recognized as a significant emerging threat to public health and food security (Food and Agriculture Organization, 2016). An alternative solution that is currently being carried out is the exploration of various natural materials to take advantage of the ability of antibacterial activity and increase livestock productivity [2-5]. Research on the combination of galangal, ginger, temulawak and honey with various concentrations has been shown to increase the productivity and carcass weight of broiler chickens. Feed supplementation with probiotics such as *Lactobacillus*, *Bacillus*, and *Clostridium* can increase growth, nutrient digestibility, and humoral immunity [6]. This research was designed to formulate a combination of ginger and honey and probiotics that has never been done before. The purpose of this study was to determine the role of the combination of ginger, honey, and probiotics (*Bacillus subtilis* and *Lactobacillus acidophilus*) as a candidate for *in vitro* AGP replacement.

Materials and Methods

The study was conducted in June 2019 on Veterinary Medicine Microbiology Laboratory, Departement of Veterinary Medicine, Gadjah Mada University. In this study, data from the disk diffusion method were analyzed by One-Way Analysis of Variance (ANOVA) and followed by the Post-Hoc Tukey test, optical density data from the dilution method were analyzed by Kruskal-Wallis and continued by the Mann-Whitney test. A significant difference from each treatment occurred when the P value <0.05. MBC determination was analyzed descriptively. Based on this framework, the following hypotheses can be formulated:

H1: The combination of curcuma and honey can inhibit the growth of *E. coli* and increase the growth of *B. subtilis* and *L. acidophillus*.

H2: The growth of *B. subtilis* and *L. acidophilus* can inhibit the growth of *E. coli*.

The research tools used were petri dishes, refrigerator, tweezers, ose, bunsen, microscope, syringe, microplate, microplate reader, digital caliper, centrifuge, and vortex. The materials used were *Eschercia coli* ATCC® 11775 (pathogen) and *Bacillus subtilis* ATCC® 6633 (probiotic) obtained from the Veterinary Center (BVET), and *Lactobacillus acidophilus* (probiotic) obtained from the collection of the UGM Inter-University Center (PAU), Yogyakarta, Indonesia, Broth Heart Infusion (BHI, MerckTM), phosphate buffered saline (pH 7.4, SigmaTM), Mueller Hilton agar (MHA, MerckTM), curcuma aquadest extract (concentration: 25%, 12.5%, 6.25%, 3.13%,

1.56%), Lombok honey (100% concentration), distilled water, blank disc (OxoidTM), chloramphenicol antibiotic disc (C 30 µg, OxoidTM).

The method used to test the antibacterial activity was Kirby-bauer disc diffusion in which the pathogenic and probiotic bacterial cultures were cultured on each medium resuspended with Phosphate Buffered Saline (pH 7,4, SigmaTM) to a concentration of 1.5 × 108 CFU/mL. A blank disc (OxoidTM) was dripped with 50 µl a combination of curcuma aquadest extract and honey then placed on the surface of MHA (MerckTM) media which had been cultured *E. coli*, *B. subtilis*, and *L. acidophilus*. Chloramphenicol disc (C 30 µg, OxoidTM) was used as a positive control, while sterile distilled water was used as a negative control and then incubated at 37°C for 24 hours. Observations were made by measuring the diameter of the inhibition zone and the growth zone around the disc. The zone of inhibition (clear zone) surrounding the disc describes the antibacterial activity of natural substances against pathogens, while the growth zone around the disc indicates the ability of natural ingredients to support the growth of probiotic candidates.

The next method is dilution to calculate optical density values on 96-well microplates on a round basis described in Clinical and Standard Institute M07-A9 and Clinical Institute Standard Institute. The combined extract with a volume of 25 µl (in 100 µl broth media, BHI for *E. coli* and *B. subtilis* culture media and MRS broth for *L. acidophilus* culture media) was placed in the first well, and then doubled dilution was carried out in each subsequent well until the lowest concentration of 1.56%. Ten microliters of each bacterial suspension with a concentration of 1.5 × 108 CFU/mL were added to each well. The broth media with various concentrations of herbal extracts was used as a negative control, while the broth media cultured with each bacterium with a concentration of 1.5 × 108 CFU/mL was used as a positive control [7]. The microplate was incubated at 37°C for 24 hours then the optical density value of the culture media was read on a microplate reader with a wavelength of 570 nm. Determination of MIC is the result of four times dilution from the initial concentration of the selected combination extract which is calculated for the optical density value. The optical density value which indicated the number 0.00 nm for *E. coli* was then cultured on MHA (MerckTM) media to determine MBC. MBC was determined using the lawning technique (spread method). The selected combination extract solution based on the results of the MIC test was taken from the microdilution plate well as much as 100 µl then spread on 15 ml of prepared MHA (MerckTM) media and then incubated at 37°C for 24 hours. Agar medium that is not covered with bacteria is designated as MBC.

Probiotics antagonist test against pathogens is also done by disc diffusion method. Probiotic candidate isolates that had previously been cultured in broth media (MerckTM) were then resuspended to a concentration of 1.5 × 108 CFU/ml, then cultured on MHA media (MerckTM) using the pour agar method [8]. 20 µl suspension of *B. subtilis*, *L. acidophilus* and their combination with a concentration of 1 × 106 CFU/ml was dropped onto a blank disc (OxoidTM). Positive control used is Chloramphenicol disc (C 30 µg, OxoidTM) while the disc is dripped PBS as a solvent used as a negative control suspension. The media was incubated at 37°C for 24 hours and the diameter of the inhibition zone (mm) was measured. All test methods were repeated three times to minimize biased results.

Results

Curcuma aqueduct extract with various concentrations combined with Lombok honey. This combination is expected to produce a combination of extract formulations that work synergistically in order to produce the best effect on bacterial growth. The combination of curcuma extracts is divided into 5 groups, combination of extracts 1: 25% aquadest extract +Lombok honey; extract combination 2: 12.5% aquadest extract+Lombok honey; combination of extracts 3: 6.25% aquadest extract+Lombok honey; extract combination 4: 3.13% aquadest extract+Lombok honey; extract combination 5: 1.56% distilled water extract+Lombok honey. The test results on the growth of the three bacteria are presented in (Tables 1-3).

Type of bacteria	Curcuma aquadest extract concentration	Inhibition zone diameter (mm)		
		Lombok Honey (100%)	Control (+) Ch. Antibiotic	Control (-) Aquadest
E. coli	0.25	8.53 ± 0.03	13 ± 0.03	<6
	12.5 %	7.34 ± 0.02		
	6.25%	6.65 ± 0.02		
	3.13%	6.95 ± 0.04		
	1.56%	6.96 ± 0.03		
B. subtilis	0.25	<6	14.58 ± 0.03	<6
	12.5 %	<6		
	6.25%	6.94 ± 0.02		
	3.13%	7.03 ± 0.02		
	1.56%	7.06 ± 0.02		
L. acidophilus	0.25	<6	14.91 ± 0.04	<6

	12.5 %	<6		
	6.25%	<6		
	3.13%	<6		
	1.56%	<6		

Table 1: The results of the antibacterial activity of the combination of aquadest *curcuma* extract and honey against *E. coli*.

ANOVA					
Escherchia coli					
	Sum of squares	**df**	**Mean square**	**F**	**Sig.**
Between groups	11.009	21	0.524	550.066	0
Within groups	0.042	44	0.001	-	-
Total	11.051	65	-	-	-

Table 2: Statistical analysis of Anova One-Way disc diffusion test results of a combination of *curcuma aquadest* extract and honey on the growth of *E. coli*.

Multiple Comparisons						
***E scherchia coli* Tukey HSD**						
(I) Herbs extract	(J) Herbs extract	Mean difference (I-J)	Std. Error	Sig.	95% confidence interval	
					Lower bound	Upper bound
Combination extract M2+TW2	Combination extract M2+TW3	1.19	0.02521	0	1.0936	1.2864
	Combination extract M2+TW4	1.88	0.02521	0	1.7836	1.9764
	Combination extract M2+TW5	1.58333	0.02521	0	1.487	1.6797
	Combination extract M2+TW6	1.57667	0.02521	0	1.4803	1.673

Table 3: Statistical analysis of Tukey's Post-Hoc test results of the combination of curcuma aquadest extract and honey on the growth of *E. coli.*

The results of the disk diffusion test showed that the diameter of the inhibition zone between the combination treatments of the concentration of aquadest extracts of curcuma and honey was significantly different (P<0.5). The combination of 25% *curcuma aquadest* extract+100% Lombok honey showed the largest inhibitory zone against E. coli and the growth zone of candidate probiotics *B. subtilis* and *L. acidophilus*, on the other hand, the positive control indicated the presence of an inhibitory zone against the probiotic candidate (Figures 1 and 2).

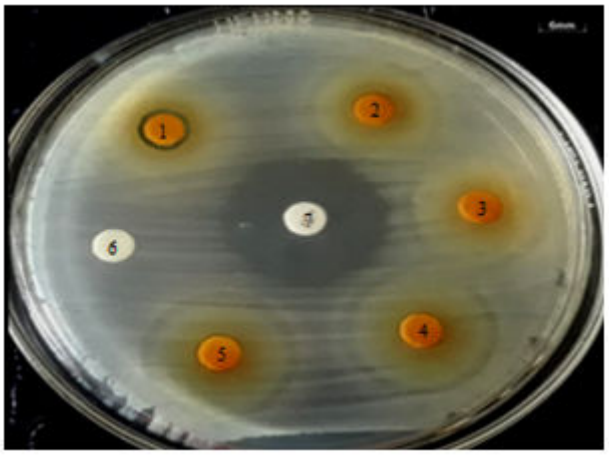

Figure 1: The results of the disc diffusion test of a combination of aquadest Curcuma extract+Lombok honey with a concentration of extract combination 1 (1), extract combination 2 (2), extract combination 3 (3), extract combination 4 (4), extract combination 5 (5), negative control (6), positive control (7) against *E. coli*.

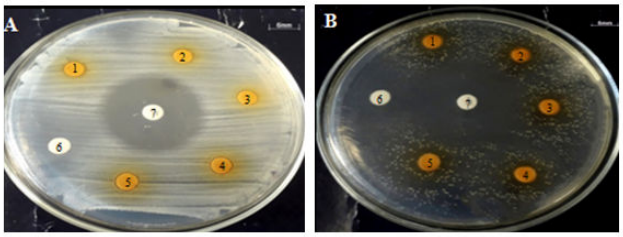

Figure 2: The results of the disc diffusion test of a combination of *Curcuma aquades* extracts of+Lombok honey with a concentration of a combination of extracts 1 (1), a combination of extracts 2 (2), a combination of extracts 3 (3), a combination of extracts 4 (4), a combination of extracts 5 (5), negative control (6), positive control (7) against *B. subtilis* (A) and *L. acidophilus* (B).

The combination that showed the largest diameter for the growth of *E. coli* and supported the growth of *B. subtilis* and *L. acidophilus* was chosen as the extract combination for which the optical density value was calculated to determine the Minimum Inhibitory Concentration (MIC) and minimum killing concentration (MBC) for the growth of *E. coli* and to see the maximum ability of the combined extract to support the growth of *B. subtilis* and *L. acidophilus* (Tables 4-7).

Combination of extracts (concentration before dilution)	**Optical density value against bacteria (nm)**		
	E. coli	*B. subtilis*	*L. acidophilus*
Lombok Honey+Curcuma aquadest extract 25%	0.00 ± 0.002	0.18 ± 0.002	0.25 ± 0.005
Lombok Honey+Curcuma aquadest extract 12,5%	0.01 ± 0.005	0.16 ± 0.004	0.25 ± 0.006
Positif control	0.15 ± 0.005	0.11 ± 0.004	0.14 ± 0.004

Table 4: Optical density values on media enriched with curcuma aquadest extract and honey against bacteria.

Kruskal-Wallis test	
Test statistics[a,b]	
	Escherchia coli
Chi-Square	46.122
Df	15
Asymp. Sig.	0
Mann-Whitney test	
Test statistics[b]	
Mann-Whitney U	0
Wilcoxon W	6
Z	-1.993
Asymp. Sig. (2-tailed)	0.046
Exact Sig. (2*(1-tailed Sig.))	0.100[1]
a. Kruskal Wallis Test, b. Grouping Variable: Ekstrak herbal, 1. Not corrected for ties.	

Table 5: Statistical analysis of Kruskal-Wallis and Mann-Whitney calculated the optical density values of herbs, honey, and probiotics on the growth of *E. coli*.

Kruskal-Wallis test
Test statistics[a,b]

	Bacillus subtilis
Chi-Square	41.961
Df	15
Asymp. Sig.	0
Mann-Whitney test	
Test statistics[b]	
Mann-Whitney U	0
Wilcoxon W	6
Z	-2.087
Asymp. Sig. (2-tailed)	0.037
Exact Sig. (2*(1-tailed Sig.))	0.100[1]
a. Kruskal Wallis Test, b. Grouping Variable: Ekstrak herbal, 1. Not corrected for ties.	

Table 6: Statistical analysis of Kruskal-Wallis and Mann-Whitney calculated the optical density values of herbs, honey, and probiotics on the growth of *B. subtilis*.

Kruskal-Wallis test	
Test statistics[a,b]	
	Lactobacillus acidophilus
Chi-Square	45.719
Df	15
Asymp. Sig.	0
Mann-Whitney test	
Test statistics[b]	
Mann-Whitney U	0
Wilcoxon W	6
Z	-1.964
Asymp. Sig. (2-tailed)	0.050
Exact Sig. (2*(1-tailed Sig.))	0.100[1]
a. Kruskal Wallis Test, b. Grouping Variable: Ekstrak herbal, 1. Not corrected for ties.	

Table 7: Statistical analysis of Kruskal-Wallis and Mann-Whitney calculated the optical density values of herbs, honey, and probiotics on the growth *L. acidophilus*.

In general, the optical density value of the combination of aquadest curcuma extract with honey which was analyzed by Kruskal-Wallis and Mann-Whitney statistics showed a significant difference ($P<0.05$) with the positive control. The calculation results explain that the combination of 12.5% curcuma aquadest extract (diluted four times to 3.13%) with 100% Lombok honey (diluted to 25%) indicates an increase in optical density value (0.01 ± 0.005) which can be interpreted that this combination of extracts is able to inhibit the growth of *E. coli* but has not been able to kill the bacteria. Different results were shown by the combination of 25% curcuma aquadest extract (diluted four times to 6.25%) with 100% Lombok honey (diluted to 25%) i.e. there was no additional optical density value (0.00 ± 0.002) which could be interpreted as the bactericidal effect works optimally on the growth of *E. coli* so that these bacteria cannot grow and develop properly. Based on the optical density values obtained, the MIC of the combination of curcuma aquadest extracts and Lombok honey is a combination of aquadest extract 3.13% +Lombok honey 25% and MBC a combination of curcuma aquadest extracts and Lombok honey is a combination of aquadest extracts of 6.25%+Lombok honey 25%. Follow-up tests were carried out to ensure and determine the Minimum Billing Concentration (MBC)

using the dispersion method on a combined extract solution from the dilution method with an optical density value of 0.00. The test results proved that the combination of 25% curcuma aqueduct extract (diluted four times to 6,25%) with 100% Lombok honey (diluted to 25%) was able to kill *E. coli* as evidenced by the absence of bacterial colony growth on agar media (MHA). The results of the MIC and MBC test results for the combination of extracts are presented in (Table 8).

Bacteria	**Combination of curcuma aquadest extract+honey (%)**	
	MIC	MBC
E. coli	*Curcuma aquadest* extract 3.13+Lombok honey 25	*Curcuma aquadest* extract 6.25+Lombok honey 25

Table 8: MIC and MBC extract combination against *E. coli*.

The addition of optical density values was also shown by the probiotic candidate's *B. subtilis* and *L. acidophilus*. The combination of 12.5% curcuma aquadest extract (diluted four times to 3.13%) with 100% Lombok honey (four times dilution to 25%) on the growth of *B. subtilis* showed a lower optical density value (0.16 ± 0.004) compared the combination of 25% aquadest curcuma extract (four times dilution to 6.25%) with 100% Lombok honey (four times diluted to 25%) (0.18 ± 0.002), while the optical density value of both herbal combinations on the growth of *L acidophilus* showed almost the same value (0.25 ± 0.005; 0.25 ± 0.006). These results illustrate the ability of the combination of curcuma extract and Lombok honey to support the growth of prospective probiotics. The synergistic effect between the two natural ingredients causes the probiotic candidates *B. subtilis* and *L. acidophilus* to grow maximally as evidenced by the higher optical density value than the positive control (media broth). The inhibitory activity of probiotic candidates was also carried out using the Kirby-Bauer diffusion method to determine the ability of *B. subtilis* and *L. acidophilus* to inhibit the growth of *E. coli*. The resulting inhibition zone diameter data can be seen in (Table 9-11 and Figure 3).

Probiotic candidate	**Diameter of inhibition zone of probiotic candidate against *E. coli* (mm)**
B. subtilis	7.18 ± 0.02
L. acidophilus	6.95 ± 0.03
Kombinasi	7.30 ± 0.02

Table 9: The results of the disk diffusion test of *B. subtilis*, *L. acidophilus* and their combination on growth *E. coli*.

ANOVA					
Escherchia coli					
	Sum of squares	df	Mean square	F	Sig.
Between groups	11.009	21	0.524	550.066	0
Within groups	0.042	44	0.001	-	-
Total	11.051	65	-	-	-

Table 10: Statistical analysis of Anova One-Way disc diffusion test results of a combination of curcuma aquadest extract and honey on the growth of *B. subtilis* and *L. acidophilus*.

Multiple Comparisons						
***Escherchia coli* Tukey HSD**						
(I) Herbs extract	(J) Herbs extract	Mean difference (I-J)	Std. Error	Sig.	95% confidence interval	
					Lower bound	Upper bound
Kombinasi probiotik	Probiotik *B. subtilis*	0.12333	0.02521	0.002	0.0270	0.2197
	Probiotik *L. acidophilus*	0.35333	0.02521	0	0.2570	0.4497

Table 11: Statistical analysis of Tukey's Post-Hoc test results of the combination of curcuma aquadest extract and honey on the growth of *B. subtilis* and *L. acidophilus.*

Figure 3: The antibacterial activity test results of candidate probiotics B. subtilis (1), *L. acidophilus* (2), combination (3), negative control (4), positive control (5) against *E. coli*.

Probiotic candidate *B. subtilis*, *L. acidophilus* and their combination showed the ability to inhibit the growth of *E. coli*. This was evidenced by the difference in the diameter of the inhibition zone of more than 6 mm around the disc containing *B. subtilis*, *L. acidophilus*, a combination of *B. subtilis*+*L. acidophilus*, and a positive control (Chloramphenicol), while the negative control showed no inhibition zone around the disc. The results of the calculation of the diameter of the inhibition zone which were analyzed with the One way Anova statistic showed a significant difference in each bacterium and also the combination of the two bacteria ($P<0.05$). *B. subtilis* showed a larger diameter of the inhibitory zone (7.18 ± 0.02) than *L. acidophillus* (6.95 ± 0.03), but the combination of the two probiotic candidates had the best inhibition zone diameter of (7.30 ± 0.02) and significantly different ($P<0.05$) compared to individual probiotic candidates.

Further testing to determine MIC and MBC against *E. coli* in this method was not carried out, because the calculation of the optical density value by the spectrophotometer was generated based on the level of turbidity of the test solution on the micro plate. The level of turbidity cannot be used to test the antagonistic ability of bacteria, because it can produce optical density values that are biased so that the determination of the antibacterial activity of probiotic candidates against *E. coli* is determined based on the diameter of the inhibition zone generated in the disk diffusion test.

Discussion

Differences in antibacterial activity of each test material can be influenced by 4 factors such as extract concentration, content of metabolites, extract diffusion power and the type of bacteria inhibited. Stated that one of the factors that influence the activity of antimicrobial substances is the concentration of antimicrobial substances [9-12]. The inhibitory power produced by antimicrobial materials will be higher if the concentration is also high. Another factor that can affect the results of the inhibition zone diameter using the Kirby-Bauer method is the ability of the extract to diffuse into the paper disc. The extract used in this test is included in the thick extract. The higher the concentration of the extract used, the higher the viscosity. The higher the viscosity of an extract, the lower the diffusion process of an antibacterial substance into the media so that it will affect the diameter of the inhibition zone.

Curcuma rhizome has a distinctive antimicrobial main compound, namely Xanthorrizol (XNT) from the terpenoid group which is larger (≥ 6%) than turmeric (≥ 3%). Curcuma generally contains antibacterial compounds which are included in the essential oil group. The antimicrobial activity of each type of essential oil can be influenced by the type and amount of active components it contains, the variety or cultivar, climatic factors and soil where it grows/area of origin, fresh or dried rhizome shape, as well as the extraction method and the type of solvent used [13-17]. Likewise, the production of other active ingredients such as terpenoid compounds in essential oils is also strongly influenced by the geographical conditions of the plant habitat as well as other specific influencing factors that are not yet known.

According to, extraction of distilled water, methanol, ethyl acetate, and n-hexane will produce a solution containing terpenoid compounds, phenols and alkaloids because the level of polarity of the solvent used is the same, from polar to semi-polar or non-polar solvents. The derivatives of phenolic compounds will interact with bacterial cells through an adsorption process that involves hydrogen bonds and can change the permeability of cell membranes [18]. Penetration of high levels of phenol into cells can cause protein coagulation and lysis of cell membranes, while low concentrations of phenolic compounds can form weak bonds and break down easily so that if phenol penetrates into cells it can cause protein coagulation and lysis of cell membranes occurs.

Another mechanism for the antibacterial role of essential oil elements is terpenoids which are thought to involve the breakdown of membranes by lipophilic components and curcumin which has a phototoxic effect on bacteria when exposed to light by producing hydrogen peroxide which can cause cytoplasmic membrane damage [19-22]. Terpenoids, phenols, and hydrogen peroxide are thought to act on bacteria by damaging the cytoplasmic membrane, this causes important inorganic ions, nucleotides, coenzymes, and amino acids to seep out of the cell, and prevent the entry of materials into the cell food or nutrients needed by bacteria to produce energy. The cytoplasmic membrane is in charge of carrying out energy metabolism in prokaryotic cells so that, if the cytoplasmic membrane is damaged, energy metabolism will not take place. This is what causes the inability of cells to grow and death cell causes.

Several studies have shown that in general, essential oils are more active against Gram-positive bacteria than Gram-negative bacteria, stated that the reaction caused by herbal phenolic compounds will affect the bacterial cell wall [23-25]. The simple arrangement of the cell wall in Gram-positive bacteria and the absence of an outer membrane cause antibacterial compounds to penetrate the cell wall and disrupt the cell wall biosynthesis process. Previous research related to the use of temulawak as a phytobiotic material has been widely carried out, but its ability to control the growth of *E. coli* (APEC) is still rarely *in vitro* studied.

The polyphenol content in herbal extracts is a compound that has high antioxidant activity to overcome free radicals and plays a role in overcoming oxidative stress generated by metabolic activity by providing a microaerophilic environment for probiotics. The content of essential oils in herbs is also able to stimulate and increase the

growth of beneficial bacteria (eg, *Lactobacilli* and *Bifidobacteria*) in the intestines. Another opinion, states that the polysaccharide component is considered the most important active immune component.

Previous research on the ability of a combination extract of temulawak extract and red ginger extract to support the growth of *L. acidophilus* was proved that the combination of red ginger ethanol extract and *temulawak aquadest* extract was able to support the growth of this probiotic candidate and increase the ability of better adhesion of pathogenic bacteria to chicken intestinal epithelial cells [26]. Another explanation regarding the mechanism of compounds in herbs that supports the results of this study was conveyed, that differences in cell wall thickness of non-pathogenic and pathogenic bacteria affect the reactions caused by phenolic compounds. The cell walls of non-pathogenic bacteria will be dehydrated so that the pores will shrink, causing the cell wall permeability and membrane function to decrease so as to minimize damage to the probiotic cell wall.

The antibacterial activity of honey can be influenced by several factors such as high sugar content, low humidity, low pH, and hydrogen peroxide. Another opinion states that antibacterial activity in honey is influenced by osmolarity, pH, activity of peroxide and non-peroxide compounds. The mechanism of antibacterial activity related to the osmolarity of honey is due to the high osmotic power of honey, because 84% of the components of honey content are glucose and fructose while water is only around 15-21%. Osmolarity causes strong interactions between sugar molecules and water molecules and leaves fewer water molecules for bacteria, making bacterial growth difficult. Another factor in the form of hydrogen peroxide contained in honey produced by the glucose oxidase process is an important component that is able to inhibit bacterial growth. Honey also contains flavonoid compounds that can damage bacterial cell walls that react with alcohol groups on flavonoid compounds so that flavonoids can enter the cell nucleus and react with DNA and cause bacterial lysis and then die. These results are also in accordance with the research of which states that bitter honey has antibacterial activity against both Gram-negative and Gram-positive bacteria.

Honey contains about 80% carbohydrates consisting of monosaccharides, polysaccharides, and oligosaccharides. The content of oligosaccharides has been widely used in various food products with the aim of being a source of prebiotics which are undigested food components and provide benefits through microbial modulation that is useful for colon health (probiotics). The prebiotic activity of local honey oligosaccharide isolates from Sumbawa had higher prebiotic activity than inulin, which is a commercial prebiotic. The synergistic effect of Manuka honey (UMF 20+) which can increase the growth of probiotics and inhibit pathogens. Normal microflora such as *Lactobacillus* and bifidobacteria will ferment oligosaccharides in indigestible honey, for the benefit of bacterial metabolism so that it can provide benefits for the host body. The prebiotic content in honey can also maintain the growth and stability of the species.

The results of this study are in line with the results of who proved that the administration of a combination herbal formulation in broiler chickens has the potential to maintain a normal microflora balance in the digestive tract. The administration of a combination of galangal, ginger, temulawak, and honey in various concentrations was proven to increase productivity and carcass weight of broiler chickens. The best increase in broiler productivity was shown after administration of a combination of herbs for 17 days with a concentration of 2.5%. Another opinion stated that probiotics can also reduce the activity of acetyl coenzyme A carboxylase, the enzyme responsible for the rate of fatty acid synthesis, by producing statins as inhibitors of fat formation in the liver. The use of probiotics supplemented with prebiotics can increase energy and protein efficiency and can reduce blood cholesterol content than the partial use of probiotics and prebiotics. The addition of probiotics and prebiotics has no negative effect on broiler chickens so that they have the same growth as chickens given antibiotics, and can even increase antioxidant activity so that fat oxidation can be inhibited.

The test of antagonistic properties between probiotics and pathogens using the Kirby-bauer diffusion method was carried out to determine the ability of *B. subtilis* and *L. acidophilus* to inhibit the growth of *E. coli*. The antibacterial activity of probiotics is influenced by several important factors [27]. The main metabolites of acidic bacteria are short-chain fatty acids and lactic acid which can inhibit the growth of pathogenic bacteria in the intestines such as *E. coli*. *Lactobacillus* aciodophilus produces two bacteriocin components, namely bacteriocin lactacin B, and acidolin which is an extracellular component in the form of peptides or compounds in the form of antimicrobial proteins that can provide an antagonistic response by inhibiting the development of pathogenic organisms. The same thing was also shown by the inhibitory mechanism of *B. subtilis* which produces antibiotics that are toxic to other microbes such as iturin A which is a lipoprotein, subtilin which is a peptide compound, and bacitracin. Bacitracin is a polypeptide that works to inhibit the formation of cell walls.

Probiotics can be a potential alternative to antibiotics to inhibit growth and reduce colonization of enteric pathogens in the intestines of poultry. Research conducted stated that *in vitro B. subtilis* has better antagonistic activity against pathogens *E. coli* O157:H7 and *S. thyphymurium* than *Lactobacilli*. The use of probiotics such as *B. subtilis*, *B. thuringiensis*, and *L. acidophilus* through drinking water was reported to be able to replace the role of antibiotics, maintain the health of the digestive tract of livestock and reduce the number of *E. coli*. Another opinion, explains that a concentration of 107-108 CFU/g of *Lactobacillus* is effective in suppressing the growth of pathogenic bacteria significantly due to a decrease in acidity or pH from lactic acid production.

Conclusion

The best extract combination formulation was determined based on the diffusion method, calculation of optical density value, determination of MIC and determination of MBC. Curcuma aquadest extract 6.25%+Lombok honey 25% (concentration after four dilutions in the dilution method) was determined as the best combination capable of killing *E. coli* and supporting the growth of *B. subtilis* and *L. acidophilus* maximally *in vitro*. Curcuma aquadest extract with a concentration of 0.39% (3.9 mg/ml) was sufficient to inhibit the growth of *E. coli* while Lombok honey required 25% (250 mg/ml) to inhibit the growth of *E. col*i. The formulation with this concentration was also able to support the growth of *B. subtilis* and *L. acidophilus*. The growth of the combination of *B. subtilis* and *L. acidophilus* probiotics was also antagonistic to the growth of *E. coli*. Based on the above, it can be concluded that the combination formulation of *curcuma aquades* extracts and honey with these concentrations can be used as a substitute for AGP.

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