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Preparation And Characterization of Bio-\Polymeric Nano Feed Incorporating Silage-Derived Organic -Acids And The Polar Fraction of Papaya Leaf Extract

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Abstract: This research has aimed to incorporate the fermented organic acids and papaya leaf extract into chitosan-alginate composites using an ionic -gelation method. The concentration of chitosan was 35 mg/7.5 mL, sodium alginate was 20 mg/7.5 mL, and cross-linking the $CaCl_2$ cross linker was 20 mM. Variations in the formula Different formulations, are carried out on organic acids with the code of referred to as kumpai-ZnO, legum-ZnO, and kumpai-legum-ZnO. These sample using various organic acids, and the formulations contained as much as 50 mg/7.5 mL of the organic acid. The results of the identification of the chemical contents of the fermented product was lactic acid derivatives as a major component, and there were no alkaloids, flavonoids, terpenoids, and or poly phenols content present. The particle characterization results showed they were a homogeneously distributed in size with PDIs from 0.199 to 0.218 with a sizes between of 253.6 to and 286.8 nm. The potential Zeta potential is was found to be -20 to -15 mV by using a particles size analyzer (PSA). Particles were spherical based on the images of transmission electron microscopy (TEM) images. The best formula formulation was is the formula 3, containing with the kumpai-legum-ZnO content, which was demonstrated by to be 85% encapsulation percentage encapsulated (%EE). The anti-*E. coli* activity test showed an increase of the particles were 40% of activity the particles compare to more active than the same substances not in particle. The ionic -gelation method successfully created a homogenous particle suspension loading loaded with the active compounds, such as the fermented organic acids and the papaya leaf extract. This system could deliver the active compounds to the bacterial cell of *E. coli* and *S. typimurium* bacterial cells.
Key words: Organic acid, chitosan, alginate, ionic -gelation, antibacterial activity

1. Introduction

Silage is one of the bioprocessing technique used for fresh materials of with fresh high- water contents in a places like (silos) under anaerobic conditions. In The process (ensilage) formed generates secondary metabolite products such as the organic acids of the fermentation process products from the conversion of carbohydrates into lactic acid by lactic acid bacteria. The more greater the amount of lactic acid bacteria, the more acid form formed in the silage. The acids that are formed are organic acids such as lactic acid, acetic acid, and butyric acid. The content of organic acids in silage derived from silage grass is higher than the acid content in the legume silage, because legumes contain higher protein contents, which can inhibit the acidification process. Previous research studies have reported that the copper-based silage resulted in higher organic acid profile content of silage made from legumes [1]. Organic acids are feed ingredients that can be used as feed additives in animal feed as a substitutes for antibiotic function because it they have antibacterial properties, which can allow them to penetrate the bacterial cell walls and disrupt the normal physiology of some types of pathogenic bacteria. Pathogenic bacteria such as *Escherichia coli* and *Salmonella typhimurium* can compete with microflora of animal in obtaining nutrients in the gastric intestinal tract, and can produce harmful metabolites products that are harmful to the host, thus which can result in impaired livestock growth and increasing the chances of contracting the disease [2]. A disadvantage of the direct administration

of organic acids in to livestock in liquid form has a disadvantage is that is not optimal in the acceptance absorption of organic acids up to the small intestine because of its their volatile nature, and they may at first initially be absorbed in the upper gastrointestinal tract (cache, gizzard and proventriculus), thus reducing their effectiveness in the small intestine and caecum. This weakness can be overcome by changing the organic acid into its salt, and then it can be delivered by a biopolymer. Organic acid salts are obtained by mixing the liquid organic acid liquid with a base to form crystals, that can which prevent evaporation and reduce organic acid metabolism in the upper gastrointestinal tract. Subsequently, Organic acid salts derived from complete maize-based corn waste material with dose of 12.5% were more effective in inhibiting *Escherichia coli* (0.33 cm) than *Salmonella typhimurium* (0.34 cm).

Papaya leaf, can be consumed as livestock feed. Papaya leaves contains flavonoids, which has antibacterial properties [3] [4] [5] [6]. Attempts have been made to extract the beneficial constituents from Papaya leaf, but the extract is less stable than the leaf so far has been tried to be extracted but the stability is less obtaining. With particle technology, papaya leaf can be diversified and made more practical the empowerment and diversification of papaya leaf use could be realized as livestock feed for preventing the growth of pathogenic bacteria. This particle technology uses polymers such as chitosan that can be destroyed in by living organisms (biodegradable) . Chitosan is not expensive biopolymer; therefore, it can be used for livestock rather than other biopolymers that more expensive pharmaceutical products. Chitosan could form particles with the presenting of in the presence of alginate and cross linking with $CaCl_2$ as a crosslinker by ionic -gelation method [7] [8] [9].

Based on the above background, the purpose of this study was to prepare, and characterize, the antibacterial activity of particles chitosan-alginate loaded with organic acids and ethanol extract of papaya leaf. The chemical content of the organic acids was performed the evaluated qualitatively test of chemical content. Against the particle was characterized the the size, distribution pattern, and homogeneity, also as well as the zeta potential (using a PSA). Particle morphology was determined with TEM images. Percentage encapsulation (% EE) was determined using UV -spectrophotometer tool. The antibacterial activity test was performed by the challenge analyzed by testing the particles against the growth of bacteria on nutrient-agar media.

2. Materials and Methods

2.1. The process of making silage

The stages of in making the silage refer to the described in a previous method report [10]. In the first step, grass of kumpai tembaga (Hymenachne acutigluma) and legume of kemon air (Neptunia oleracea Lour.) were cut with the size 2-5 cm and stored for 24 hours for the process them to withering. After that, it was weighed as many as 500- g batches of withered material were weighed out for each treatment. Each sample was mixed with molasses, which had been dissolved in water, as many as at as much as 3% by weight of forage, and then the mixture was stirred until blended. Furthermore, each treatment forage was put into transferred to a 3-layered plastic bag of 3 layers and then compacted to air compressed to remove all air. The bags were then tied tightly and stored for 21 days in a dry place and not exposed to protect from direct sunlight. After 21 days, the bags of silage were opened, and taken to be tested for testing in the laboratory.

2.2. Isolation of organic acids from the silage

The process of isolating the organic acids carried out using maceration extraction method with methanol as the solvent [11]. First of all, 50-g sample of swamp forage weighed 50 g and then was immersed into 250 ml of methanol. The sample covered with plastic wrap and coated with aluminum foil. Furthermore, it was stored on in a fume hood. Maceration was done for 3 days. Every 24 hours, the methanol extracts obtained from the silage were combined. The combination of the methanol extract was evaporated using a rotary evaporator at 50°C, with rotation speed of 60 rpm to form a viscous extract.

2.3. Dispersion of the polymer

To prepare the the chitosan preparation solution, 35 mg of chitosan powder was dissolved in 7.5 mL of 1% acetic acid solution in Beaker and then homogenized with magnetic stirer at 75 rpm for 30 min at room temperature until solution was completely dissolved. Every 20 mL of chitosan solution pipeline contains 12 mg of chitosan for each formulations. To prepare the sodium alginate solution, the 9.6 mg of sodium alginate preparation was dissolved within 60 mL of API in a glass beaker and glass then homogenized with magnetic sttirer at 75 rpm for 30 min at room temperature until sodium alginate solution was completely dissolved. Every 20 mL of sodium alginate solution pipette contains 3.2 mg for each formulation [12].

2.4. Preparation of the particles

A 50-mg sample of papaya leaf extract 50 mg was added to 7.5 mL of the chitosan solution in the a vial using a magnetic stirrer and magnetically stirred at 750 rpm [6] [8] [9] [10], and this mixture was used for each formulation and to submicron particles [13]. Sodium alginate was dispersed in aqueduct using magnetic stirrer at a rate of 750 rpm 60 $^{\circ 0}$C, and taken 20 mL of this mixture was taken as mass 2 for each formulation and as mass 2. The mixture mixing of the two masses was performed by dropping the solutions from with a 50- µL micro pipet above the a solution being magnetically stirred and then continuing to stir the solution at 750 rpm for 1 hour. Calcium chloride solution was added to avoid the rapid agglomeration [14] [15].

2.5. Purification and determination of encapsulation (%EE)

Purified sub micro particles of chitosan- sodium alginate was carred out by using 300 kDa viva spin filters and centrifugation for 15 min until 2 phases. The phases was absorbed by the Viva-spin 300 kDa filter [16]. The supernatant phase was separated, with 30 mL of Aqua Injection (API) into in the sample holder and centrifuged again. Determination of maximum wave length scanning was done at the lowest concentration solution possible using UV-Vis spectrophotometer. After that, the absorbance of solutions measurement at all concentration of a standard (quercetin). Determination of the EE% values were determined using calibration curve with concentration each from 0.10; 0.20; 0.30; 0.40 and 0.50 µg/mL solutions of standard prepared from the stock solution with a concentration of 10 µg/mL stock solution. Measurement of absorbance was done using standard solutions and the supernatant of purification were measured. EE% values were then determined based on the amount of free substance present in the solutions.

2.6. Antibacterial Activity

One Ose of each culture of test bacteria (*E. coli and S. typhimurium*) was, inoculated on the surface of the nutrient agar medium in a Petri dish by a scratch method. After that, the samples were incubated for 24 hours at 37°C. Bacterial suspensions were made using rejuvenated bacteria. Furthermore the bacteria were inoculated into 10 mL of nutrient broth medium and homogenized. After that it the homogenates were then incubated for 24 hours at 37°C. The concentrations of the extract used refers to the optimum formula obtained after the based on the percentage of efficiency of the encapsulation (% EE). Antibacterial testing was performed by diffusion method using 6- mm discs paper. Antibacterial activity was reported as shown by the diameter of the clear zone formed around the paper disc paper.

A total of 1- mL aliquot of each the suspension of the each test bacteria was inoculated into a petri dish which contained 15 mL of nutrient media. Approximately 50 µL of the test solutions were dropped on to the discs 50 L. The supply of nanoparticles was placed in a nutrient agar medium inoculated with bacteria. In each petri dish there were also negative controls (chitosan and alginate), a positive control (tetracycline (30 ppm), and papaya leaf ethanol extract (2% (w/v). The dishes were then incubated for 24 hours at 37°C. The clear zones were measured using a sliding term with total

area calculations reduced by the diameter of the paper disc diameter. Three replicates were performed for each treatment consists of three repetitions.

3. Results

The acids formed during the this process include lactic acid, acetic acid and butyric acid. Leguminous feed such as *C. calothyrsus*, with their high protein contents, such as *C. calothyrsus* can be stored using an appropriate method such as silage. However, these legumes generally have low salt contents and low buffer capacity, which cause low silage quality. In this research study, we already evaluated the contents of organic acids produced by during the fermentation of kumpai and legumes. Precipitation of the organic acids was facilitated by the hydroxides of base of Na, Ca, and Zn. The results in Table 1 shown that kumpai has more potential than the legume. Because of source of our system is based on the herbs, we also determined found that other secondary metabolites such as alkaloids, flavonoids, terpenoids, and polyphenols another compound that might could also be entrapped present in the fermentation result products such secondary metabolites alkaloid, flavonoid, terpenoids, and polyphenol. These secondary metabolites had influence to the growth of normal flora in the digestive tract of livestock. The results was that only lactic acid derivatives were only available as the precipitation salts of the organic acids.

Papaya plants produce fruit that is with known to benefits, but the leaves can still be increased its more efficiently used use. Papaya leaves are commonly used consumed as vegetables and are also used as animal feed. Papaya leaves have a high fiber and flavonoid contents that can counteract the growth of pathogenic microbes. Papaya leaves contain semi polar to polar active compounds, that are in the fraction of semi polar to polar while the nonpolar fraction contains terpenoids compounds and waxes. The results regarding of to the screening photochemistry phytochemical studies on the of extract papaya leaf extracts was shown in Table 1 and Figure 1. Based on previous report, besides so far papaya leaf is in addition to being a fodder, papaya leaf is also then a good nutrient delivery system required by the farm for livestock. So that the This means that organic acids from the fermentation process can be used as a probiotics for the support of good beneficial bacteria, such as *Lactococcus*, in the digestive system, that is *Lactococcus* and the acids can be then followed by papaya extract, to which can handle the pathogenic bacterial pathogens. Livestock have the Healthy digestive systems ensure the propertion there for the supply of nutrients will be guarantee livestock.

Table 1. Identification of The Fermentation Products and Phytochemical Tests of papaya leaves

Identification	Result
Protein test	-
Lipid test	-
Organic acid test	+++
Disacharide test	-
Polysaccharide test	-
$CHCl_3 + H_2SO_4$ -	-
$FeCl_3$ test	+
Dragendorff test	++
Cyanidin test	++

Described mark : The symbols indicate the following: - (revealed showed no reaction), , + (revealed color by reaction showed a color change in the reaction), +++ (revealed color by very strong reaction showed a very strong color change)

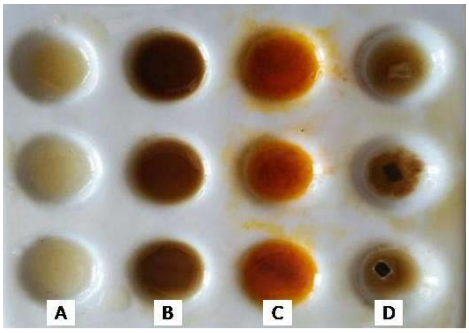


Figure 1. Image of Colors_Observed in the Phytochemistry Tests Steroids Test (A) Polyphefol Test (B) Alkaloid Test (C) and Flavonoid Test (D)

Table 2. Inhibition Zone Nanoparticle Feed From Silage

Bacteria	Green Silage	*Inhibition Zone (cm)*			
		----------*Doses ZnO (%)*----------			*Avarage*
		12,5	17,5	22,5	
E. coli^{in}	P1	0,39 ± 0,01	0,31 ± 0,06	0,24 ± 0,04	0,32^{b}
	P2	0,32 ±0,08	0,33 ± 0,08	0,28 ± 0,10	0,31^{b}
	P3	0,22 ± 0,03	0,27 ± 0,06	0,23 ± 0,03	0,23^{a}
	Average	0.30	0,31	0,25	
S. typhi*	P1	0,22 ±0,03	0,32 ±0,03	0,15 ±0,01	0,22^{b}
	P2	0,08 ±0,03	0,14 ±0,04	0,14 ±0,02	0,12^{a}
	P3	0,10 ±0,05	0,12 ±0,03	0,12 ±0,04	0,11^{a}
	Average	0,13^{a}	0,19^{b}	0,12^{a}	

Explanation : Silver Kumpai (P1), Silver Kumpai + Water Kemon (P2), Water Kemon (P3). Significant Effect of Interaction (*). Unsignificant Effect of Interaction (^{tn}). The same Superscript in the same colum and row showed significance difference (P<0,05).

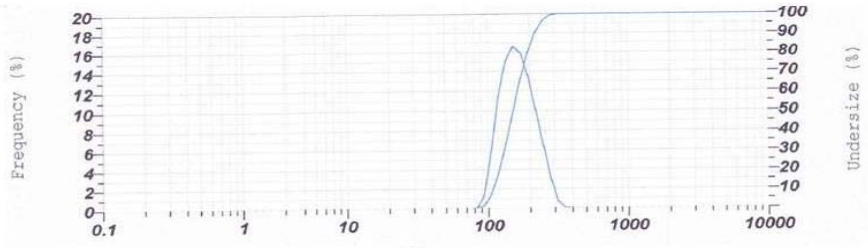
From statistical data, showed that interaction between green silage and ZnO dose effect unsignificantly (P<0,05), aganits inhibition zone of *E. coli*. ZnO concentration in this research can not give good impact on inhibition zone aganist *E. coli.* Concentration organic acid from silage significantly enhance inhibition zone against *E. coli.* Organic acid has known as antimicrobial which is

potential to inhibit patogen [17]. P3 showed the lowest result compare with other treatment. P3 was composed by water kemon legum which is contain hight protein concentration. Hight protein concentration can effect to the BAL growth due to ensilase process and gave negatif impact on organic acid production. According to Ali *et al.* (2012), water legum kemon contain crude protein about 28,02%. Hight protein could declaine acidify process, and inhibit the growth of BAL [18].

Due to variance analyses, there was significant interaction (P<0,05), between green silage and ZnO concentartion against avarage inhibition zone against *S. typhimurium.* From further test result P1 has give the best result from other treatment, and with concentration 17,5% organic acid and the existence of ZnO, can inhibit the growth of *S. typhimurium.*

Combination of low pH with organic acid (especially lactic acid) act as important aspect to inhibit patogen bacteria [19]. Lactic acid can inhibit *S. typhimurium* because of low pH and intraselluler activity of bacteria sel [20]. According to Sharma et al (2017), Zn as micro mineral play as antibacterial mineral. ZnO can disrupt bacterial membranne, alter the permeability of membrane and can induce oxidative stress on bacteria and inhibit cell growth [21].

The polymers that support the ionic-gelation loading papaya leaf extract and acid derivatives through-derivated ionic gelation are chitosan and alginate. Positive charges on the chitosan many benefits can beneficially can be mainly interacted interact with the negatively charged alginate. First, chitosan is interacts with the papaya leaf extract, and then this interaction the newly formed complex is coated with alginate. Alginate can make bonds with calcium chloride. Therefore, on the outer layer of the particles, we can also put append the fermented organic acids from the fermentation for the legumes and kumpai as probiotics to form NPs loading loaded with 85% of probiotics and papaya extract. The size, PDI, and zeta potential also as well as the TEM images of the particles was shown in Figures 2 and -3. The conclusions of the arrangement of ultimately, materials in the particles we designed are, in the surface layer there is a probiotic, and the coating contains coated in there substances that can inhibit the growth of pathogenic bacteria. In some Previous research, it studies have indicated that papaya leaves did not cause a problem to inhibit the growth of normal flora, so that the cattle eat well can these leaves with no negative side effects. Probiotics could also suppress the growth of pathogenic bacteria as shown in Table 2 and Figure 4. In the results of this study, when it the materials were incorporated into became particles, there was a notable increase in activity a visible activity increase compared to nanoparticles extract papaya leaf extract not in particle form.



2A

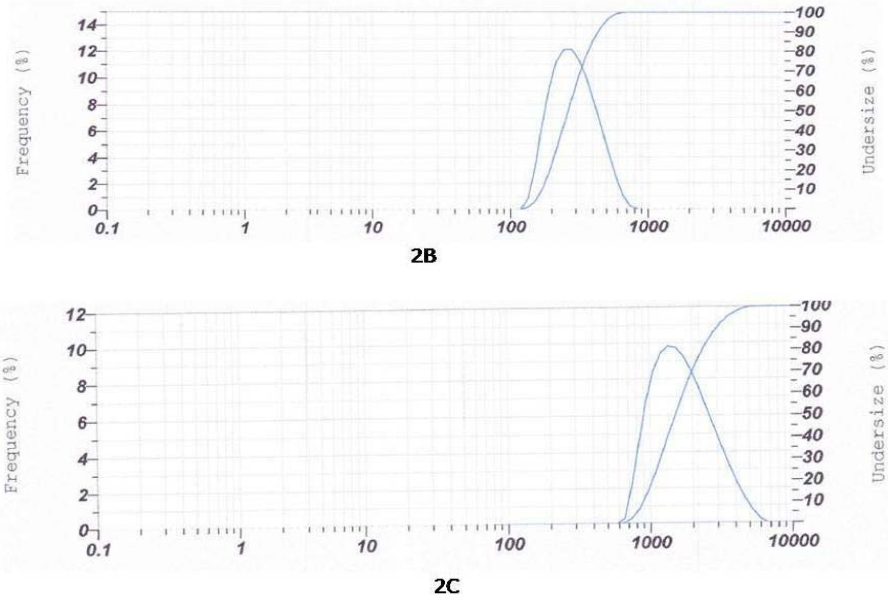


Figure 2. Size of the Particles and Their PDIs with at concentrations of 12.5 (A) 17.5 (B) and 22.5 (C).

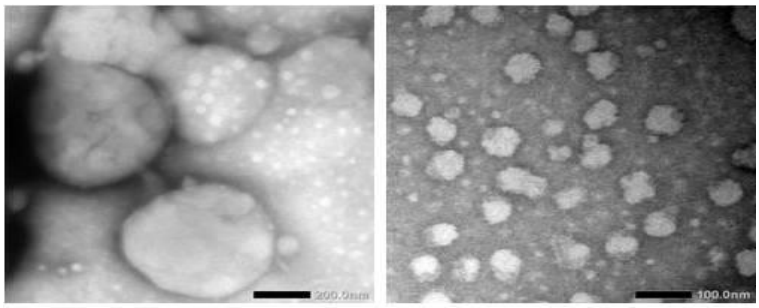


Figure 3. Transmission electron microscopy (TEM) Images.

Table 3. Trials Tests of Pathogenic Bacteria Against Organic Acid Salts from Swamp Forage Silage

Type of silage	Base	*concentration*	*Escherichia coli* (cm)				*Salmonella typhimurium* (cm)			
			1	2	3	average	1	2	3	average
Kumpai	NaOH	12.5	0.1	0.2	0.1	0.13	0.2	0.15	0.12	0.16
		17.5	0.2	0.15	0.15	0.17	0.13	0.1	0.12	0.12
		22.5	0.1	0.2	0.1	0.13	0.3	0.3	0.2	0.27
	$Ca(OH)_2$	12.5	0.1	0.3	0.1	0.17	0.2	0.3	0.15	0.22
		17.5	0.15	0.1	0.3	0.18	0.15	0.1	0.25	0.17
		22.5	0.1	0.15	0.1	0.12	0.1	0.13	0.12	0.12

		12.5	0.38	0.4	0.39	**0.39**	0.23	0.27	0.23	0.24
	ZnO	17.5	0.25	0.37	0.3	0.31	0.15	0.15	0.1	0.13
		22.5	0.28	0.25	0.2	0.24	0.3	0.35	0.3	**0.32**
		12.5	0.1	0.05	0.1	0.08	0.1	0.1	0.2	0.13
Legume	NaOH	17.5	0.1	0.15	0.1	0.12	0.25	0.22	0.2	0.22
		22.5	0.1	0.1	0.1	0.10	0.13	0.1	0.15	0.13
		12.5	0.15	0.15	0.1	0.13	0.12	0.2	0.2	0.17
	$Ca(OH)_2$	17.5	0.2	0.1	0.1	0.13	0.1	0.2	0.1	0.13
		22.5	0.15	0.2	0.2	0.18	0.1	0.12	0.2	0.14
		12.5	0.25	0.4	0.3	0.32	0.1	0.1	0.05	0.08
	ZnO	17.5	0.35	0.4	0.25	0.33	0.14	0.12	0.15	0.14
		22.5	0.25	0.2	0.4	0.28	0.1	0.13	0.18	0.14
		12.5	0.15	0.1	0.1	0.12	0.2	0.2	0.25	0.22
Kumpai-Legume	NaOH	17.5	0.1	0.1	0.1	0.10	0.15	0.2	0.15	0.17
		22.5	0.1	0.1	0.1	0.10	0.25	0.25	0.15	0.22
		12.5	0.1	0.25	0.1	0.15	0.2	0.15	0.2	0.18
	$Ca(OH)_2$	17.5	0.2	0.15	0.15	0.17	0.15	0.15	0.1	0.13
		22.5	0.1	0.1	0.1	0.10	0.15	0.2	0.15	0.17
		12.5	0.2	0.2	0.25	0.22	0.15	0.1	0.05	0.10
	ZnO	17.5	0.3	0.3	0.2	0.27	0.15	0.08	0.12	0.12
		22.5	0.25	0.25	0.2	0.23	0.15	0.1	0.1	0.12

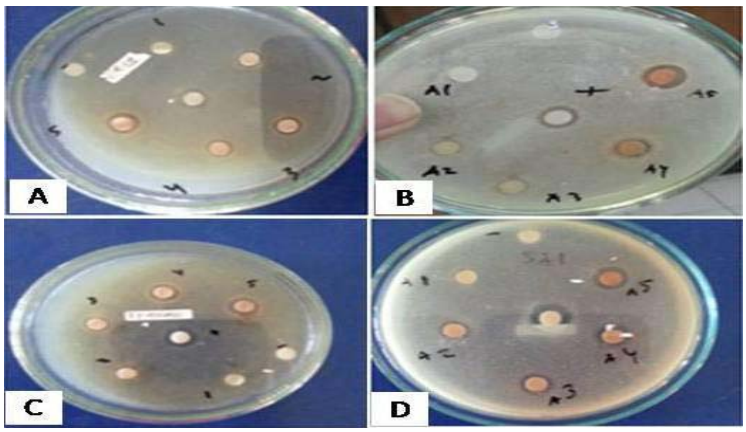


Figure 4. The growth of *E. coli* by NPs-eks activity (A). *S. Typhimurium* by NPs-eks activity (B). *E. coli* by NPs-org Ac-eks activity (C). and *S. Typhimurium* by NPs-org Ac-eks activity (D)

4. Discussion

Silage is a fodder produced by the fermentation of materials with high water contents fermentation process. The High-quality of the silage is achieved when lactic acid as the dominant acid is produced as the dominant acid; a rapid decrease in pH indicates, an efficient acid fermentation is occurring when the pH silage decrease occurs rapidly. The faster the fermentation occurs, the more nutrients can be retained contained in the silage can be obtained. *Enterococcus faecium* EF9296 type can be used as an inoculum because it can be used to suppress the work of pathogenic bacteria such as *Salmonella spp*. It can also be used to maintain the environmental ecosystem of the silage and the quality of microbiological quality during the fermentation process [27].

There are two ways of making silage, by chemically and biologically. The chemical way is carried out by method involves the addition of acids as preservatives such as formic acid, propionic acid, hydrochloric acid and sulfuric acid as preservatives. The addition is required for to rapidly decrease the pH of the silage to descend immediately (to about 4 approximately 4.2). This condition will inhibits respiration, proteolysis and prevents the activation of Clostridia bacteria. While in biologically method, by fermenting the ingredients are fermented until the naturally formed acid is formed that decreases the pH of the silage. The identification of the chemical contents of the fermented product was indicated the presence of lactic acid derivatives, and there were no alkaloids, flavonoids, terpenoids, and poly phenols content present. The polar substances, such as lactic acid derivatives, are is suitable for interacting with the chitosan encapsulated by alginates. This lactic acid derivatives are a food source that fully nourish normal flora in cattle rumen. If normal flora is fertile leads to the livestock digestion will also be being optimal for fiber processing because of normal flora will produce hydrolytic enzymes and decompose the fiber breaking regime into nutrients that enter the body of the livestock. These nutrients will have a direct effect on muscle weight and livestock health. [22]

Nutrition in livestock is generally absorbed through a mechanical process by involving the organs of the livestock because it depends on the size of the substance to be being eaten and requires diminution of in size. Many creations prepared nanoparticles production have been tried tested primarily for targeted drug delivery, but that were not many few of them have been applied to increase livestock productivity. This research is the first step to develop more broaden the potential from applicability of an extracts from Indonesia to for enhancing the productivity of livestock. Natural botanical extracts is herbal ingredients that, they must have been proven safe for humans, will also be safe for livestock. For nutrient delivery systems, the effects of mechanical processes need to be reduced minimized by reducing the length duration of the process of chewing and rumen destruction processes [23]

With the an emphasis of on the overlap between nanoparticle technology that also has touched and the world of farms agriculture, then this there is an opportunity to create the ideal particles form and certainly that are also biodegradable. Nano encapsulation systems of for polar compounds such as lactic acid derivatives have long been experimented studied and are often formed by researchers to be made into small objects particles for rapid absorption in the intestines. Nano precipitation Starting from the is a precipitation technique in requiring two mixed solvents with the but the polymer is being more soluble in one than the other, and the product precipitates out precipitation in the form of nanoparticles, this method is known as Nano precipitation [27]. There is another method that uses a water-insoluble polymer as that is soluble in oil and, therefore may be able to form it might be an emulsion form. The emulsion occurs when one or even two phases are by emulsified one or even two stages. When the oil solvent evaporates, the polymer will be suspended as into a nanometer- sized particles;, this method is known as emulsion solvent evaporation. The opposite thing is In contrast to this is a the simple method such as ionic gelation; because the polymers used in this method are polar, and most natural materials such as acid derivatives, flavonoids, and saponin are more polar or semi polar, so it they are better suited to fits with this system. In addition, polymers such as chitosan that

can be used for in ionic gelation systems are polymers that are numerous common and easy to find in Indonesia. For the foreseeable future, the assurance of bbioactivity test and toxicity test of the prebiotic and extra-unnatural materials in the nanoparticles needs to be done conducted soon to gain a full and become the material of its own understanding of the ingredients in the various extracts of Indonesian natural ingredients products [25]

As the In conclusions the production produced Nano feed has been successfully characterized and showed to be homogeneous in distributed size with PDIs between 0.199 to and 0.218 with a size and sized of 253.6 to 286.8 nm. The potential Zeta potential was found to be between -20 to and -15 mV by using particle size analyzer (PSA). Particles were spherical based on the images of transmission electron microscopy (TEM) images. The best formulation is the formula 3, which contains with the kumpai-legum-ZnO content, which was demonstrated by and was found to be 85% encapsulation percentage encapsulated (%EE) by using based on UV -spectrophometers. The anti-*E. coli* activity test showed an increase of the particles were 40% of activity the particles compare to nor active than the same active substances not in particle active substances [26]

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