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Research article

Effects of *Achatina fulica* mucus as an antimicrobial additive on chemical compositions, fermentation quality, and *in vitro* digestibility of elephant grass silage

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Abstract

This study was purposed to evaluate the utilization of *Achatina fulica* mucus as antimicrobial to increase the quality of elephant grass silage. Total of 100 mL of *Achatina* mucus (AM) was isolated from 20 head *Achatina fulica* using electric shock method. The AM contained 3,07 mg/5 mL of total protein. The 45 d old of elephant grass was harvested, wilted, and then chopped at 3-5 cm. Chopped grass was ensiled into 4 kg mini-silo for 21 d with different silage additives in triplicate, consisting of: without additives (P0); with 1% of AM (P1); and with combination of AM and microbial complex inoculant at 1:1 ratio (P2). The compositions of elephant grass silage were not affected by application of additives. The P2 silage resulted in the lowest ($P<0.05$) pH and yeast count. Both P1 and P2 silages had higher ($P<0.05$) lactate concentration than P0. Moreover, clostridia was not detected in both P1 and P2 silages. Both P1 and P2 silages had a higher ($P<0.05$) in vitro digestibility of dry matter and organic matter than P0 silage. The present study concluded that applying AM was effective to inhibit clostridia and improve fermentation quality and digestibility in the rumen. The application of P2 was generally recommended to increase the quality and digestibility of elephant grass silage.

Keywords: Achatina fulica mucus, Achacin, Additive, Antimicrobial, Elephant grass, Silage

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INTRODUCTION

The dry season results in low production of forage in several areas of tropical countries, including Indonesia. Limitation of water causes a lack of forage growth and lead to decrease the ruminant production. The preservation of forage is the way to sustain the ruminant industry during the dry season. Silage is a common and easy method to provide forage by reducing the pH of lactic acid bacteria (LAB) during the fermentation period. Silage will break down the cellulose into simpler sugar, which is easy to metabolize (Hanh and Tham, 2023).

Tropical climate is a new challenge to make good quality silage. High temperatures during ensiling potentially increase the growth of undesirable microbes, such as yeast, mold, and clostridia (McDonald et al., 1991; Bernandes et al., 2018). Clostridia is the most undesirable microbe that contaminates tropical areas. Moreover, it could easily to growth in high-moisture grass (Paradhipta et al., 2019). The optimum temperature for fungal and clostridia growth is 25°C to 37°C (Bernandes et al., 2018), similar to climate conditions in tropical areas. Clostridia contamination increases butyrate concentration and decreases the palatability and nutrient content of silage (McDonald et al., 1991; Driehuis et al., 2018). In addition, clostridia has a positive correlation with other undesirable microbes, such as yeast and mold. Contamination of fungal increases nutrient loss and produce toxic substances (McDonald et al., 1991; Alonso et al., 2013). The development of natural antimicrobial additives as an alternative way to reduce clostridia contamination is still in a limited study.

An antimicrobial peptide (AMP) is a small protein that acts as the first barrier of defense against microbes and discovered in many plant and animal species, such as plants (thionins), amphibians (magainin), crustaceans (callinectin), and mammals (lactoferrins) (Sarkar et al., 2021). Giant African Snail (*Achatina fulica*) is the most widespread and invasive terrestrial snail pest which has a high potency to produce AMP called achacin (Lima et al., 2020). Achacin is a small molecular-weight glycoprotein with broad-spectrum antimicrobial activity in fungi, viruses, and bacteria including clostridia (Zhong et al., 2013; Dolashki et al., 2020), which are expected to inhibit the contaminant in silage. Achacin isolated from the body surface mucus has anti-bacterial properties against both gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative (*Escherichia coli*). Achacin inhibits the bacterial strains when they are growing phase (Otsuka-Fuchino et al., 1992). This study aims to investigate the effect of *Achatina* mucus (AM) on chemical compositions and fermentation quality of elephant grass silage as a single additive or combined with microbial complex inoculant (MCI). The present study would be the basic research for the development of AM as a silage additive in further study.

MATERIALS AND METHODS

Mucus preparation

The research was conducted in Yogyakarta, Indonesia (-7.767149358573889, 110.38584313068287). Snails which was length 5-10 cm and weight about 30 – 40 g were collected from the agriculture area as

a pets. The AM samples were obtained from 20 head of *A. fulica* snails, by electric shock at 5 – 10 volts for 30 – 60 sec. Total 100 mL of AM was obtained form 20 head of snails. The collected mucus was used as silage additive. The measurement of total protein on AM was conducted to predict the concentration of achacin as antimicrobial substance. The AM was macerated with water at ratio 1 : 2 for 24 h at 4°C. The water soluble fraction (WSF) of mucus is obtained by mixing water : mucus at 2 : 1 ratio. The obtained liquid was mixed with pure ethanol at 1 : 3 ratio and then centrifuged at 2.900 *g* for 30 min. The supernatant was collected after centrifugation. The total protein of collected supernatant was determined using the Lowry method (Mæhre et al., 2018). The collection of AM and determination of total protein were conducted in three different period with different population of *A. fulica* snails. The determination of total protein was purposed to ensure that collected AM contain protein which might be an achacin.

Silage production

Harvested of elephant grass (*Pennisetum purpureum*) at 45 d was wilted for 2 d to reach an optimum moisture content (65% – 70%) for silage production according to previous studies (Lee et al., 2019; Paradhipta et al., 2021). The forage was copper to a length about 3 – 5 cm. The chopped forage was sub-sampled at 500 g for chemical composition analysis. The forage was ensiled into 6 kg mini-silo for 21 d with different silage additives in quadruplicate, following: P0, silage without MA; P1 silage with 1% of MA; P2, silage with AM and MCI at ratio 1:1. The MCI used product of *Effective Microorganisms* 4 (EM4; Bali, Indonesia), which contained mixture of *Lactobacillus* sp., *Saccharomyces cerevisiae*, and *Rhodopseudomonas palustris at* 10^6 *colony forming unit* (cfu)/mL, respectively. After ensiling for 21 d, each silage was sub-sampled at 500 g for analyses of chemical composition. Other, silage was also sub-sampled approximately at 20 g for silage extraction. The silage extraction was used for analyses of fermentation characteristics and microbial counts.

Chemical compositions

The sub-sampled fresh forage and silage were dried at 105ºC for 24 h to measure the dry matter (DM) concentration method (method number 934.01; AOAC, 2005). Sub-sampled silage was dried at 60ºC for 48 h, and ground using a cutting mill to pass through a 1 mm screen. The crude ash concentration (CA) was determined using a muffle furnace at 550ºC for 5 h (method number 942.05; AOAC, 2005). The crude protein (CP) and ether extract (EE) concentrations were analyzed by the Kjeldahl method (method number 984.13; AOAC, 2005) and the Soxhlet method (method number 920.39; AOAC, 2005), respectively. The neutral detergent fiber (NDF) (method number 2002.04; AOAC, 2005) and acid detergent fiber analysed (ADF) (method number 973.13; AOAC, 2005) were analysed using ANKOM. The Non-fiber carbohydrate (NFC) was calculated according to Cherney et al. (2003).

Fermentation characteristics

Total 20 g of sub-sampled silage was blended with 180 mL of ultra-pure distilled water for 30 sec to make silage extraction. This silage extraction was used to analyses of pH and microbial counts. The pH was measured using pH meter. The concentration of ammonia-N was determined following the procedure of (Apriyanti et al., 2013) using an Ultraviolet-Visible (UV-VIS)

spectrophotometer and based on the Indonesian National Standard (SNI) 06-6989.30-2005. Meanwhile, concentration of lactate was determined according to procedure of Barker and Summerson (1941) using UV-VIS spectrophotometer. The concentrations of acetate, propionate, and butyrate were determined using gas chromatography (Aglient 5977B Series MSD, USA) with FFAP capillary column 30 m × 250 µm × 250 µm (Quadrex Corporation) according to procedure of Filipek and Dvorak (2009). The carrier used nitrogen with 1.0 mL/ min as flow. The detector used FID with temperature program was 60°C – 200°C. The injection size of sample was 1 µL using automatic device. Limit of detedtion (LOD) and limit of quantification (LOQ) were estimated using chromatograms of samples spiked at the lowest level validated. The LOD (ppm) were determined about 6.023, 13.109, and 13.995 for acetate acid, propionate acid, and butyrate acid respectively. Meanwhile the LOQ (ppm) were determined about 18.249, 39.723, and 42.409 for acetate acid, propionate acid, and butyrate acid, respectively. The data of ammonia-N, lactate, acetate, propionate, and butyrate were expressed as % of DM (%, DM).

Microbiological counts

Silage extraction was continued into several dilution series consisting of 10^{-2} to 10^{-8}. Several medium was prepared to analyse the enumeration of LAB, yeast, mold, and clostridia. The LAB was cultivated on de Man, Rogosa, and Sharpe agar (MRS; Sigma Aldrich, USA). Yeast and mold were grown on Potato Dextrose Agar (PDA; Sigma Aldrich, USA), and Clostridia on the Reinforced Clostridial Agar (RCA; Himedia, India). All plates of MRS agar were incubated anaerobically at 30°C for 48 h, while all plates of PDA and RCA were incubated aerobically at 30°C for 48 h according to previous studies (Lee et al., 2019; Paradhipta et al., 2021). Colony counts were presented as cfu per gram of silage, and then transformed to log10.

In vitro ruminal digestibility

The rumen fluid was collected from two cannulated Bali cattle *(Bos indicus)* before morning feeding. The Bali cattle weighting 300 kg were fed rice straw and commercial concentrate mixed at a 7:3 ratio (CP 12% and ME 10 kcal/kg). The animal care and procedure for *in vitro* study was approved by The Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Gadjah Mada (No 025/EC-FKH/Eks./2023). Two layers of cheesecloth was prepared to filter a collected rumen fluid. Rumen fluid was mixed with anaerobic culture medium at a 1:4 ratio as described by Tilley and Terry (1963). The mixture of rumen fluid and anaerobic culture medium was called rumen buffer. The ground sample was weighted at 500 mg and then put into an incubation bottle. Rumen buffer at 40 mL was added into each incubation bottle. To reach anaerobic conditions, all incubation bottles were gassed with CO_2 and closed tightly. Each treatment used quadruplicate using 4 bottles along with four blanks. All incubation bottles were placed at 39°C in an incubator. The *in vitro* incubation was conducted in two batch as replication of period. After 48 h of incubation, all incubation bottles were opened and the remain sample was calculated to determine *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD) following the procedure of previous studies (Tilley and Terry, 1963; Paradhipta et al., 2022). The rumen buffer from each bottle was collected to determine a rumen pH using the pH meter (Paradhipta et al., 2021).

Organoleptic appearances

At 21 day of ensiling, silage was observed organoleptic appearances, such as color, aroma/odor, and texture according to previous study (Tahuk, 2020). Total of 20 non-expert panelists filled in the question form according to several indicator. The indicators of organoleptic appearances consisting of color (dark, young green, brownish green), aroma/odor (neutral, sourish, sour), texture (slimy, damp, dampish). The data of organoleptic appearance would be used to support the Principal Component Analysis (PCA).

Statistical analysis

The research was carried out based on a completely randomized design (CRD) with three treatments and three treatment replications. Data were analysed using one-way analysis of variance (ANOVA) with SPSS IBM 20, if there was a significant difference between treatments ($P < 0.05$) continued with Duncan's test with a significance level of 5%. Its general model was:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} = response variable, μ = overall mean, T_i = the effect of additives, e_{ij} = error term. Interrelationship among various variables of silage quality were investigated using PCA. There silage variable consisted of organoleptic appearance, organic acids, pH, ammonia, and microbe profiles. The direction and length of the vector are interpreted the relationship among silage variables. The PCA was performance by R Software using a Facto Mine R Package according to procedure of Lê et al. (2008).

RESULTS

Total protein of *Achatina* mucus

The measurement was conducted in three different periods. In period 1, the total protein of AM was 2,98 mg/5 mL. In period 2 and 3, the total proteins of AM were 3,10 and 3,15 mg/5 mL, respectively. Generally, AM contains an average of 3.07 mg/5 mL of total protein (Table 1). This result indicated that collected AM potentially contain achacin.

Table 1 The concentration of crude achacin protein in *Achatina* mucus

Time	Concentration (mg/5 mL)
Period 1	2.98
Period 2	3.10
Period 3	3.15
Average	3.07

Chemical compositions

In general, concentration of DM, CA, CP, EE, NDF, ADF, and NFC from the elephant grass that used in this study were 32.5%, 16.7%, 8.77%, 2.56%, 63.3%, 40.6%, 8.67% respectively (Table 2). After ensiling, concentrations of DM, CA, CP, EE, NDF, ADF, and NFC from the silage were not affected by application of additives (Table 3). In general, the average concentrations of DM, CA, CP, EE, NDF, ADF, NFC from all treatments were 28.9%, 15.6%, 7.05%, 1.92%, 60.95%, 44.17%, and 14.6% respectively.

Table 2 Chemical compositions of fresh forage before ensiling (%, DM)

Item	
Dry Matter	32.5 ± 1.05
Crude ash	16.7 ± 1.10
Crude protein	8.77 ± 0.21
Ether extract	2.56 ± 0.13
Neutral detergent fiber	63.3 ± 0.80
Acid detergent fiber	40.6 ± 0.90
Non-fiber carbohydrates	8.67±2.24

Table 3 Effects of *Achatina* mucus on the chemical composition of (%, DM)

Item	Treatments			P-value
	P0	P1	P2	
Dry Matter	29.4 ± 0.97	29.1 ± 2.62	28.2 ± 4.21	0.956
Crude ash	15.7 ± 0.95	15.6 ± 0.44	15.5 ± 1.61	0.997
Crude protein	6.54 ± 0.31	6.89 ± 0.67	7.71 ± 0.24	0.254
Ether extract	2.13 ± 0.27	2.09 ± 0.36	1.55 ± 0.20	0.427
Neutral detergent fiber	64.7 ± 1.33	60.4 ± 2.06	57.7 ± 1.62	0.109
Acid detergent fiber	42.3 ± 2.26	41.5 ± 1.38	38.7 ± 1.08	0.330
Non-fiber carbohydrates	11.2±2.86	15.0±3.53	17.5±3.67	0.488

[1] P0, silage without additive addition; P1, silage with 1% mucus addition; P3, silase with 1% mucus + 1% MCI addition.

Fermentation characteristics

Application of P2 resulted in the lowest (P=0,009; 4,74 vs. 5,27 and 5,27) pH of silage (Table 4). The concertation of ammonia-N was not affected by application of additives in the present study. The average of ammonia-N concentration from all treatments was 0,253%. In addition, concentrations of lactate (P=0,001; 3,62% and 4,91% vs. 1,50%) and acetate (P=0,006; 1.55% and 1.65% vs. 1.05%) were higher in P1 and P2 compared to P0. The concentration of propionate was not affected by application of additives. The butyrate was not detected in P1 and P2, while it was presented in P0 (p=0.007; ND and ND vs. 0.08%).

Table 4 Effects of *Achatina* mucus as silage additive on fermentation characteristics of silage

Item	Treatments			P-value
	P0	P1	P2	
pH	5.36^{a} ± 0.16	5.19^{a} ± 0.04	4.74^{b} ± 0.13	0.010
Ammonia-N, % DM	0.24 ± 0.02	0.23 ± 0.02	0.21 ± 0.03	0.629
Lactate, % DM	1.50^{b} ± 0.62	3.62^{a} ± 0.86	4.91^{a} ± 0.16	0.001
Acetate, % DM	1.05^{b} ± 0.22	1.55^{a} ± 0.06	1.65^{a} ± 0.13	0.006
Propionate, % DM	0.41 ± 0.90	0.39 ± 0.11	0.39 ± 0.10	0.964
Butyrate, % DM	0.08^{a} ± 0.04	ND	ND	0.007
Lactate : acetate	1.41^{b} ± 0.43	2.36^{ab} ± 0.56	3.03^{a} ± 0.30	0.012

[a.b] Different superscripts show significant results (p<0.05).

P0, silage without additive addition; P1, silage with 1% mucus addition; P3, silase with 1% mucus + 1% MCI addition.

P0, silage without additive addition; P1, silage with 1% mucus addition; P3, silase with 1% mucus + 1% MCI addition.

Microbiological counts

Application of P1 and P2 resulted in lower (P=0.001; ND and ND vs. 4,08 log10 cfu/g) clostridia count than P0 (Table 5). On the other side, yeast count was lower (P=0.001; ND vs. 4,05 and 4,00 log10 cfu/g) in P2 than P0 and P1. The LAB count did not affected by silage additive. In general, mold was not detected in the present study.

Table 5 Effects of *Achatina* mucus as silage additive on microbial counts (log10 cfu/g)

Item	Treatments			p-value
	P0	P1	P2	
Lactic acid bacteria	5.08 ± 0.06	5.03 ± 0.19	5.17 ± 0.06	0.454
Clostridia	4.08[a] ± 0.06	ND[b]	ND[b]	0.001
Yeast	4.05[a] ± 0.08	4.00[a] ± 0.01	ND[b]	0.001
Mold	ND	ND	ND	NA

[a.b] Different superscripts show significant results (P<0.05).
[1] P0, silage without additive addition; P1, silage with 1% mucus addition; P3, silase with 1% mucus + 1% MCI addition.
ND, not detected (< 10^3 log10 cfu/g).

In vitro ruminal digestibility

Application of P1 and P2 resulted in higher IVDMD (P<0.05; 46.6% and 46.9% vs. 44.8%) and IVOMD (P<0.05; 62.3% and 62.6% vs. 60.7%) than P0 (Figure 1). On the other side, the rumen pH after 48 h of incubation was affected by treatments in the present study. Application of P1 and P2 also presented a lower (P<0.05; 6.49 and 6.49 vs. 6.52) rumen pH than P0 (Figure 2).

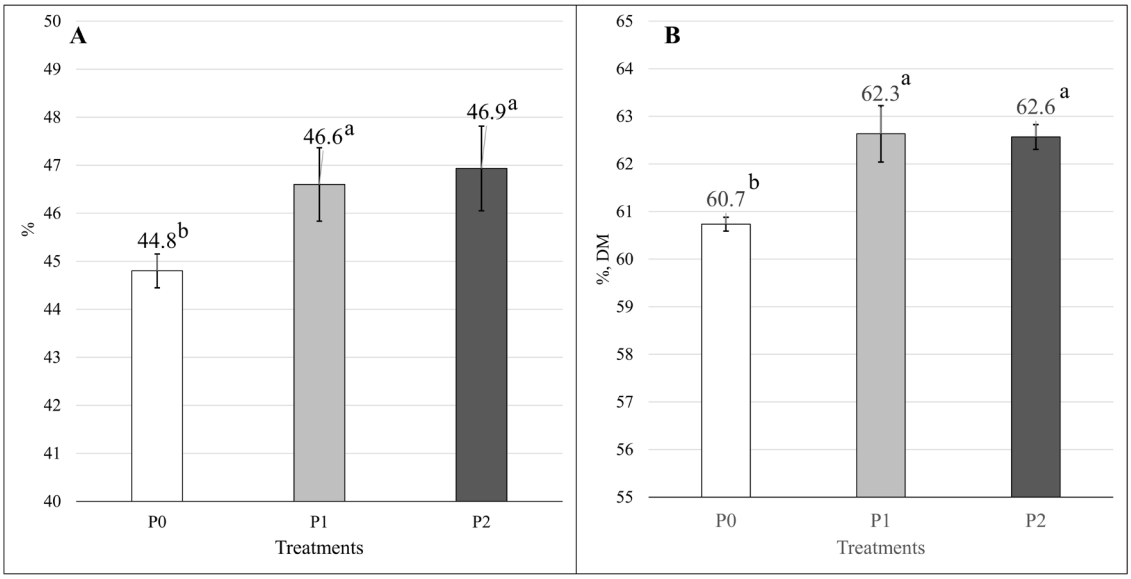

Figure 1 *In vitro* dry matter digestibility (A) and *in vitro* organic matter digestibility (B) of elephant grass silage treated with different additives. P0, silage without additive addition; P1, silage with 1% mucus addition; P3, silase with 1% mucus + 1% MCI addition. [a.b] Different superscripts show significant results (p<0.05).

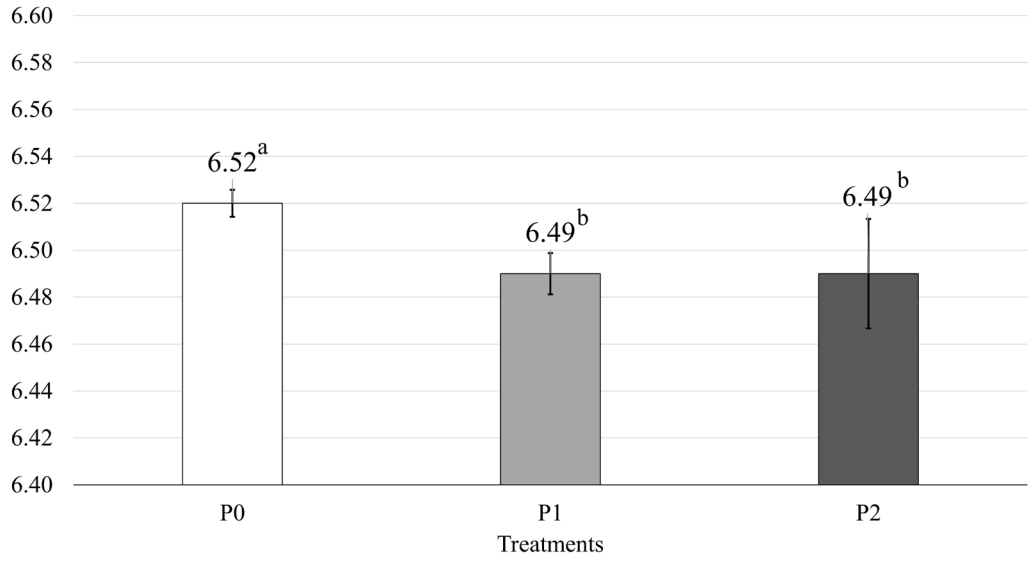

Figure 2 Rumen pH after ruminal incubation of elephant grass silage treated with different additives. P0, silage without additive addition; P1, silage with 1% mucus addition; P3, silase with 1% mucus + 1% MCI addition. [a.b] Different superscripts show significant results ($p<0.05$).

Organoleptic appearances

Organoleptic appearance of silage consisted of color, aroma, and texture (Figure 3). The color of silage mainly was brownish green in P1 and P2. Total 75% of respondents were identified a silage color as brownish green in P1 silage, while total 85% of respondents were identified same in P2 silage. In P0 silage the color was identified as brownish green by 50% of respondents and brown by 50% of respondents. The aroma of P0 silage was sourish by 60% of respondents, acid by 25% of respondents, and rotten by 15% of respondents. The aroma of P1 silage was sourish by 50% of respondents, acid by 40% of respondents, and rotten by 10% of respondents. The aroma of P1 silage was acid by 55% of respondents, sourish by 45% of respondents, and rotten by 5% of respondents. The dampest silage was in P0 silage, followed by P1 silage, and the P2 silage according to the respondents.

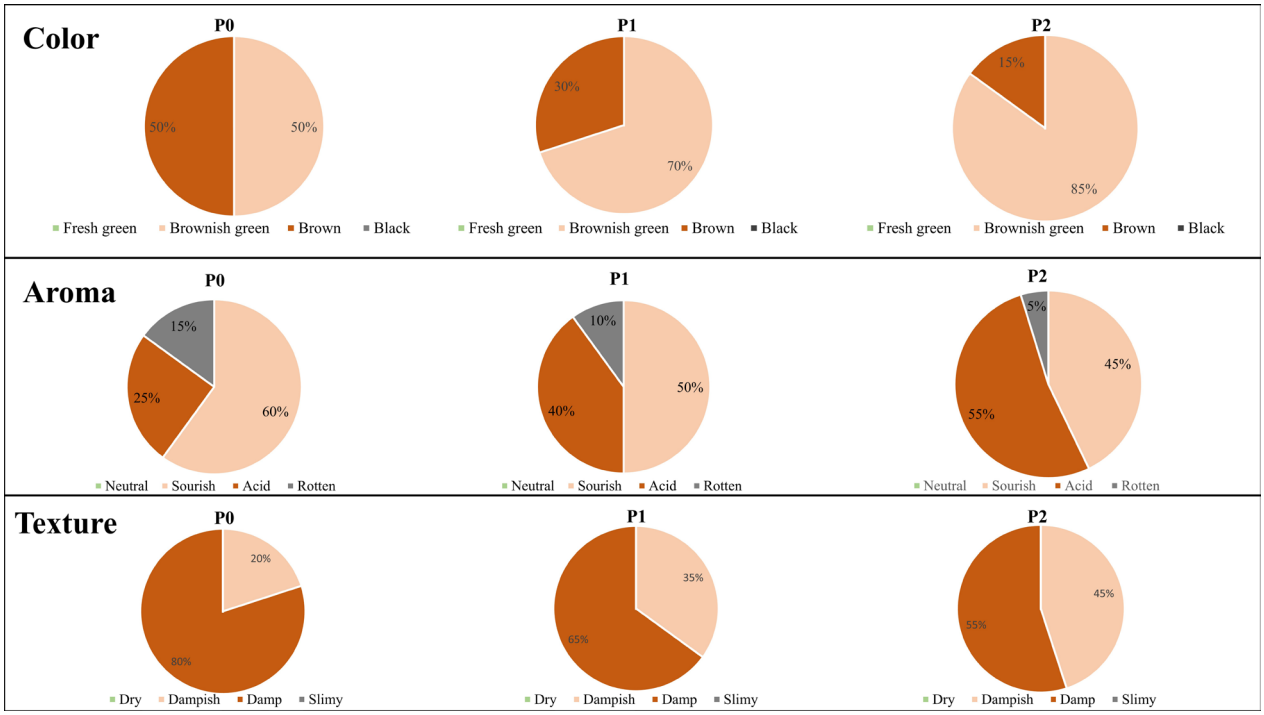

Figure 3 Color, aroma, and texture of elephant grass silage treated with different additives. P0, silage without additive addition; P1, silage with 1% mucus addition; P3, silase with 1% mucus + 1% MCI addition.

Interrelationship between parameters

The interrelationship between parameters was analyzed using PCA (Figure 4). The result of the first principal component (PC 1) was 65,4% of total variation. In PC 1, high positive loading values (>0.6) was reported in LAB, acetate aroma/odor, texture, color, and lactate. In other side, the high negative loading values (>-0.6) was reported in clostridia, ammonia-N, pH, yeast, propionate, and butyrate.

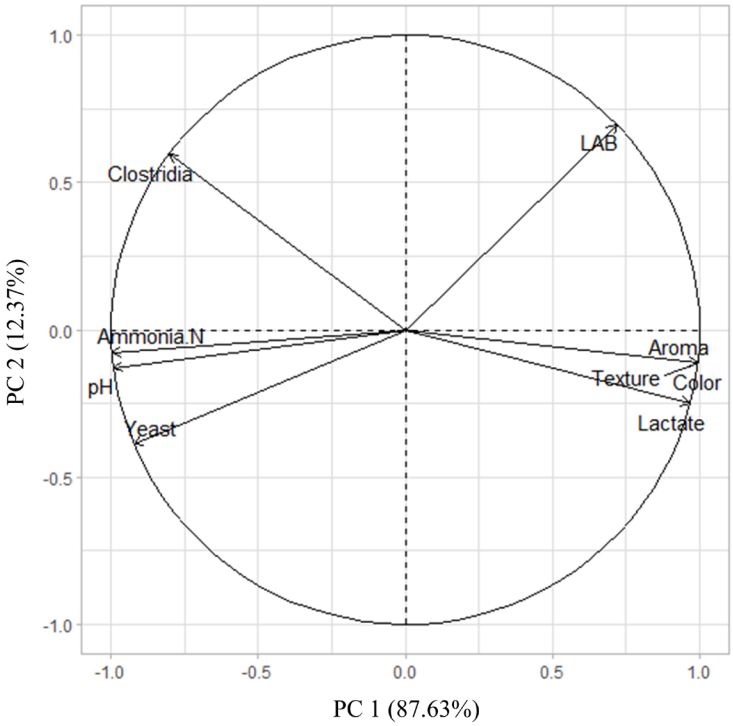

Figure 4 Principal component biplots of silage variables. LAB, lactic acid bacteria; PC 1, first principal component; PC 2, second principal component.

DISCUSSION

Molluscs have two general defense reactions are cellular defense reactions and reaction medicated by humoral defense factors. Cellular defense factor is a somatic cell system that protects against foreign pathogens and encapsulates foreign materials (Ito et al., 2011). The AMPs from AM are small protein molecules that have broad antimicrobial activity against undesirable bacteria such as *Escherichia coli*, *Clostridia perfingens*, and *Pseudomonas aureofaciens* (Dolashki et al., 2020). Most AMPs have a wide range of microbial activity against gram-positive and gram-negative bacteria and yeasts (Zasloff, 2002). Many antimicrobial peptides have been found from mucus of the snail of *Achatina fulica* such as achacin and mytimacin (Mafranenda et al., 2014). Achacin is considered to contribute to the snail's innate immunity, but the mechanism by which it does so has not been determined (Ehara et al., 2002). Achacin is the most abundant mucus protein secreted by the body's collar. Achacin belongs to the flavin enzyme family of amine oxidases, including L-amino acid oxidase which result in K-ketoacids, hydrogen peroxide, and ammonia. Some L-amino acid oxidase have antimicrobial properties (Stiles et al., 1991). Achacin (purified or recombinantly expressed) inhibited growth of gram-negative and gram-positive bacteria, and it was shown to attack the surface membrane of bacteria but not directly cause lysis of bacterial cells (Otsuka-Fuchino et al., 1993; Lima et al. 2020). A novel cysteine-rich AMP (mytimacin-AF) belonging to the peptide family of mytimacins is purified and characterized from the mucus of the *Achatina fulica* snail. Mytimacin-AF is effective against a wide variety of microbes, with gram-positive bacteria.

The collected of AM in this study presented a total protein approximately at 3,07 mg/5 mL. This indicated that the collected AM had a potential containing antimicrobial to inhibit the undesirable microbe in silage, mainly such as clostridia.

In the present study, chemical compositions of fresh forage were in the normal range according to previous studies (Bezerra et al., 2019; Costa et al., 2020). Ensiling process can decrease concentration because LAB use it to produce organic acid. Moreover, the low quality of ensiling will lead a nutrient loss, such as protein (McDonald et al., 1991). Ensiling of silage is influenced by so many factors such as sugar content, buffering capacity, dry matter, types of microbial inoculant, temperature during fermentation, rate of harvest, air exposure during harvest, and storage influence the fermentation and preservation of forage as silage (Rajabi et al., 2017). In general, addition of additives had no chemical compositions of elephant grass silage. This result of present study was in agreement with previous studies (Paradhipta et al., 2020, Paradhipta et al., 2021, Lee et al., 2021), which application of silage additive did not always affect chemical composition. The ensiling process in all silage was optimum to reduce the nutrient loss during storage. It also could be indicated by similar ammonia-N concentration in all silages (Table 4). Ammonia-N is one of indicator for protein loss during ensiling (McDonald et al., 1991; Kung et al., 2018).

In the fermentation characteristics, the combination of AM and MCI resulted the lowest pH of silage. The pH is affected by concentrations of organic acid and ammonia-N. High concentration of lactate and acetate with lower concentration of ammonia-N results in lower pH condition of silage (Kung et al., 2018, Paradhipta et al., 2020; Paradhipta et al., 2021). Lactate has high acidification value to reduce the pH rapidly (McDonald et al., 1991). Mainly, the lowest pH of P2 silage was caused by high lactate concentration (Table 4). The EM4 contain LAB, which could stimulate the lactate production and then resulted a better ensiling quality in P2. In general, pH in all treatments were above the recommended pH for silage. This could be caused due to low water soluble carbohydrate (WSC) content in elephant grass. In the further study, addition of WSC is mandatorily applied for silage production using tropical grass such as elephant grass. On the other side, the use of single AM also resulted in the improvement of fermentation quality such as increases of lactate and acetate with the decease of butyrate. These results indicated that the use of AM might stimulate the fermentation. The detail mechanism was unknown yet. However, the antimicrobial of AM might inhibit an undesirable microbe to growth, which help the LAB to produce organic acids optimally. Achacin is given to cells that are resting or growing, and it only has bactericidal (not bacteriostatic) activity against bacteria that are actively growing. This activity is comparable to that of the beta-lactam group of antibiotics, which prevent the production of peptidoglycan on the surface of cells. Achacin ought to eliminate these two types of bacteria by targeting their shared components, such as the peptidoglycan layer, components of cytoplasm, and cytoplasmic membrane (Otsuka-Fuchino et al., 1992).

The concentration of ammonia-N had no effect by silage additives in the present study. This result is supported by the result of CP concentration (Table 3), where the concentration of CP was similar in all silages. The

ammonia-N reflect the protein loss during ensiling, which could be caused by undesirable microbes (McDonald et al., 1991; Paradhipta et al., 2019). Furthermore, the CP concentration of the silage increases, resulting in the increase of ammonia-N production (Andika et al., 2015) The decrease in pH reduce effect on ammonia-N because it does not occur protein hydrolysis. Lower the ammonia-N concentration of silage results in a better silage quality (McDonald et al., 1991; Kung et al., 2018; Paradhipta et al., 2021). Ammonia concentrations are high due to excessive protein breakdown, a slow decline in pH and the growth of *Clostridium* or enterobacteria (McDonald et al., 1991; Bernandes et al., 2018; Kung et al., 2018).

The concentrations of lactate and acetate were increased by silage additives, both in P1 and P2 silages. Application of AM on silage was reported by the present study to increase the ensiling quality of elephant grass. The butyrate concentration also decreased or not detected in P1 and P2, which supported the results of clostridia growth in the present study (Tabel 5). Butyrate is an indication of bad fermentation process and the present of clostridia (McDonald et al., 1991). In addition, The AM was confirmed to have antimicrobial to inhibit the undesirable microbes, such as clostridia (Table 5). The reduce of undesirable microbe could help to improve ensiling quality by increasing lactate production (McDonald et al., 1991). Moreover, mixture of AM and MCI had no negative effects on lactate production. It indicated that antimicrobial from AM did not inhibit the LAB growth from EM4 during ensiling. It could be seen that the application of P2 results the high production of organic acids (lactate and acetate) and numerically increase population LAB of silage.

In microbial count, the antimicrobial of AM in P1 and P2 silages effectively inhibited the growth of clostridia. The result of present study was in agreement of Muck et al. (2018). According to Muck et al. (2018), the development of silage additives should consider the inhibition of clostridia, not only yeast or mold. Clostridial fermentation can increase the proteolysis and DM loss of silage (Muck et al., 2018). However, the antimicrobial of AM did not yet effective to inhibit the growth of yeast. Yeast was still detected in P1 silage. Yeast can be inhibited by acid condition during ensiling (McDonald et al., 1991; Sucu et al., 2016; Muck et al., 2018). In the present study, only P2 silage had pH lower than 5, which might be a proper condition to inhibit yeast growth. High production of lactate in P2 silage (Table 4) could decrease silage pH rapidly, which directly inhibited the undesirable microbe (Paradhipta et al., 2020). The presence of mold in the diet could be harmful for animals. Mold could produce toxicants that have negative effects for animal health (Alonso et al., 2013). The findings of this investigation demonstrate that the production of organic acid, specifically lactate 1.50% – 4.91%, exhibited sufficient inhibitory effects on mold growth in all silage samples. The findings of this investigation resulted in an accelerated and enhanced fermentation process. Li et al. (2019) reported that the lactate acid concentration range of 0.027% – 0.60% is necessary to effectively suppress microbial growth. According to the findings of Kung et al. (2018), it was shown that a yeast and mold population lower than 10^6 cfu/g had the potential to lead to increased aerobic stability.

Application of AM presented a beneficial effect on rumen digestibility by improvement of IVDMD and IVOMD. The results of IVDMD and IVOMD

also supported by lower rumen pH, which indicated a higher degradation of substrate. High degradation of substrate in rumen could produce a higher volatile fatty acid concentration that decrease a rumen pH (Hobson and Stewart, 1997). The improvement of IVDMD and IVOMD could be due to the antimicrobial activity of AM that inhibit the undesirable microbe in the rumen and increase the digestibility of substrate (Hobson and Stewart, 1997).

The results of organoleptic appearances of silage was supported the result of fermentation quality and microbial counts. Based on the biplots of PCA (Figure 4) for microbial counts, fermentation characteristics, and organoleptic presented that PC1 explains 65.4% and PC2 19.6% of the variation in the data. PC1 and PC2 had a linear positive correlation between butyrate, propionate, ammonia-N and negative correlation of LAB and acetate. The clostridia had negative correlation of texture, color, aroma, and lactate. In addition to pH and yeast had positive correlation. The organoleptic appearances such as color, aroma/odor, and texture had a positive relationship with lactate and LAB count. High count of LAB could produce high concentration of lactate, which could improve the organoleptic appearance of silage. According to previous studies, the high population of LAB was increase the organic acid concentration, such as lactate (McDonald et al., 1991; Kung et al., 2018; Muck et al., 2018). In the present study, an increase of lactate concentration resulted in brownish green color, sour smell, and dampish texture. The low quality of silage results dark color, fishy or ammonia-like odor, and slimy texture. Kung et al. (2018) reported that the concentration of lactate could influence the organoleptic appearance of silage. Clostridia, ammonia-N, pH, and yeast had a negative relationship with LAB, lactate, and organoleptic appearances in the present study, which were in agreement with previous studies (McDonald et al., 1991; Kung et al., 2018; Muck et al., 2018). Clostridia and yeast increase the nutrient loss and produce ammonia-N and butyrate with could result bad odor of silage (Kung et al., 2018).

CONCLUSIONS

Application of AM as single or combined with MCI has no effect on chemical compositions of elephant grass silage. However, application of AM confirms to inhibit the growth clostridia and improve the fermentation characteristics and ruminal digestibility of elephant grass silage. The AM do not inhibit the growth of LAB from MCI during ensiling, which could be proven by beneficial effects on fermentation characteristics and the reductions of butyrate, clostridia, and yeast.

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