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Research Article
Swamp Forage Silage Organic Acid Profiles and Influence of Silage Liquid Organic Acid Salts Against Pathogenic Bacteria *In vitro*

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Abstract

Background and Objective: Swamp forages are a potentially good quality poultry feed source and organic acids produced by fermentation determine the quality of the silage as a dietary source. This study was conducted to determine the organic acid profile in swamp forage silage and the effect of silage liquid organic acid salts on pathogenic bacteria drag zones. **Materials and Methods:** A completely randomized design was used consisting of threes wamp forage silage treatment groups with five replications: P1 (100% *Hymenachne acutigluma*), P2 (50%:50% *H. acutigluma*: *Neptunia oleracea Lour*) and P3 (100% *N. oleracea Lour*). The silage with the best organic acid profile was then reacted with NaOH (pH 10-12), $Ca(OH)_2$ (pH 4.6-4.8) and ZnO (pH 4.6-4.8) to produce organic acid salts. Total lactic acid bacteria, acidity (total amount and pH), organic acidprofiles (lactic, acetic and butyric acid), total organic acid salt and pathogenic bacteria (*Escherichia coli* and *Salmonella typhimurium*) in drag zones after *in vitro* treatment with organic acid salt extracts were measured. **Results:** P1 silage had the highest total lactic acid bacteria, total acid and lactic acid content ($p<0.05$) and lowest butyric acid levels ($p>0.05$). Though not significantly different between treatments, acetic and butyric acid levels of all silages exceeded standards for good quality. Addition of ZnO to P1 silage liquid produced the most organic acid salt ($p<0.05$ versus NaOH) with the lowest pH ($p<0.05$ versus NaOH and $Ca(OH)_2$]. ZnO-derived organic acid salt extracts produced the largest drag zone (greatest growth inhibition) for both *E. coli* [$p<0.05$ versus NaOH and $Ca(OH)_2$] and *S. Typhimurium* ($p<0.05$ versus NaOH), while NaOH produced the smallest. **Conclusion:** *Hymenachne acutigluma* (P1) swamp silage had the best organic acid profile due to a higher amount of active lactic acid bacteria. Furthermore, addition of 12.5% ZnO to P1 silage liquid resulted in organic salt with the lowest pH and optimal inhibition of *E. coli* and *S. typhimurium* growth. These results indicate that *Hymenachne* swamp forage silage can be used as an alternative feed additive (organic acid salt) which is good for poultry.

Key words: Organic acids, organic acid salts, swamp forage silage, drag zone, liquid, poultry feed

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Swamp forage has great potential as a source of food for poultry because of its high availability and good nutritional value. Some species of swamp plants in Indonesia, such as kumpaitembaga (*Hymenachne acutigluma*) and dan kemon air (*Neptunia oleracea Lour*), contain soluble carbohydrates and other potential bacterial substrates, enabling the production of good quality silage[1]. Organic acids produced by fermentation determine the quality of the silage as a dietary source. High-quality forage silages typically contain 1.50-2.50% lactic acid, 0.50-0.80% acetic acid and 0.10% butyric acid[2]. Lactic acid bacteria (LAB) ferment water-soluble carbohydrates to produce lactic acid, while acetic acid typically results from secondary fermentation of amino acid carbon chains[3]. Butyric acid, on the other hand, is largely the result of *Clostridium* sp. activity.

Organic acids produced from silage fermentation processes can be used as acidifying feed additives. The ability of organic acids to pass through bacterial cell walls enables them to have both pro- and antibacterial properties by stabilizing gut microflora and reducing/inhibiting pathogenic bacteria in the digestive tract[4]. However, it is important to note that these beneficial effects are precluded when organic acids are metabolized and absorbed in the front gut, gizzard and proventriculus (organic acids are not well-metabolized or absorbed in the duodenum and cecum)[4]. Organic acid salts result from combining organic acids and alkaline (basic) substances, which reduce evaporation and organic acid metabolization in the front gut[5]. The purpose of the present study was to determine the organic acid profile in swamp forage silage and the effect of silage liquid organic acid salts on pathogenic bacteria drag zones.

MATERIALS AND METHODS

Experimental design: This study was conducted using threes wamp forage silage treatment groups with five replications. The treatments were: P1 (100% *H. acutigluma*), P2 (50%:50% *H. acutigluma*: *N. oleracea Lour*) and P3 (100% *N. oleracea Lour*). The silage with the best organic acid profile was then reacted with base compounds NaOH (pH 10-12), $Ca(OH)_2$ (pH 4.6-4.8) and ZnO (pH 4.6-4.8) to produce organic acid salts. The research variables included total LAB content and acidity (pH) based on the method described by Fardiaz[6]. Organic acid profiles were determined using gas chromatography according to Yulianis *et al.*[7]; organic acid salts and their effects on inhibitory zones of pathogenic bacteria were measured using the method of Negara *et al.*[5].

Silage production and organic acid extraction: Silage was produced according to the previous method[5]. First, the *Hymenachne* and *Neptunia* forages were cut to size (2-5 cm long) and withered for 24 h. Cut and withered forage samples (500 g per sample)were then mixed with molasses, dissolved in water (3% of the forages) and mixed thoroughly. The mixture was then used to produce silage put into three layers of plastic bags which were compacted to remove all air. Finally, bagged samples were stored for 21 days at room temperature in a dry place and not exposed to direct sunlight. Silage samples were then analyzed for total LAB content, acidity (pH) and organic acid content.

Organic acids were extracted from bagged silage samples using methanol extraction[8]. Bagged silage samples (50 g) were immersed in a 250 mL methanol solution, covered with plastic wrap, coated with aluminum foil and stored under a hood for 3 days. Methanol extracts were collected from samples every 24 h and combined with the previous fraction for each. After a 3 day maceration period, methanol extracts were evaporated using a rotary evaporator at 50°C with a rotation speed of 60 rpm to form viscous organic acid extracts.

Organic acid salt production from silage liquid: The silage liquid sampling was carried out according to the Negara *et al.* method[5]. In the present study, none of the silage produced liquid (effluent). Therefore, silage was rinsed with distilled water to produce silage in a 1:1ratio,and the liquid obtained was put into a cloth bag to further filter out large solids. The liquid was then centrifuged at 2000 rpm for 15 min to remove residual biomass from the liquid; the supernatant was used to produce organic acid salts. Organic acid salts were obtained by reacting liquid silage containing organic acids with a base [12.5% NaOH, $Ca(OH)_2$, or ZnO]. After reacting, samples were stored for 24 h at room temperature. Then, samples were centrifuged at 2000 rpm for 15 min to sediment the salt. Salts were dried in an oven at 60°C for 3-7 days. Dry organic acid salts were ground into a fine powder manually using a mortar grinder.

Statistical analysis: The data were presented as the mean±standard deviation of five experimental replicates. The data were analyzed using one-way analysis of variance and continued with least significant difference (LSD) test set at 5% if there were differences between the treatments. Values of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Total LAB content, acidity and organic acid profiles of swamp forage silage: Silage produced from swamp forages, kumpaitembaga grass (*Hymenachne acutigluma*) and kemon air legume (*Neptunia oleracea Loui*) had significant effect ($p<0.05$) on the amount of LAB. The amount of LAB of *Hymenachne silage* (P1) was significantly higher than other treatments. (8.24 ± 0.28 log CFU mL^{-1}, Table 1). This is likely due to the high carbohydrate content found in *Hymenachne* grass (25.21-28.21% cellulose, 33.59-35.43% hemicellulose and 11.44-11.7% lignin)[1]. Homofermentative LAB species can convert 95% of glucose and other hexose sugars into lactic acid, small amounts of CO_2 and other volatile acids (e.g. but yric and acetic acid) during silage fermentation. Carbohydrate-rich substrates, such as *Hymenachne* grass, accelerate LAB fermentation, create more total organic acid, thus accelerate the decrease in pH of silage[9]. Furthermore, the same results were found in an other study in which the carbohydrate content of the material was able to support the growth of LAB; thus, it has sufficient nutrients for subsequent regeneration process[10].

The total organic acid content in P1 silage (3.80 ± 0.67%) was also significantly higher ($p<0.05$) than all of the other treatments, while the total acid content of P2 and P3 silage was not significantly different ($p>0.05$; Table 1). The high total organic acid content within P1 is also most likely due to the high soluble carbohydrate content of this grass, which largely derives from nitrogen-free extracts (46.76%); *Neptunia* legumes only have 44.86% nitrogen-free extract content. Nitrogen-free extracts (sugars, starches, etc.) are an indispensable energy source for almost all bacteria, especially LAB. Therefore, higher nitrogen-free extract leads to a higher total acid content in the product[11]. *Neptunia* legumes, on the other hand, had the high protein content (28.02%[1] versus 6.21-8.97% in *Hymenachne*[12]).Thus, P2 and P3 silage samples had lower total LAB content, resulting in less fermentation and subsequent organic acid production (Table 1).

Of the three organic acids analyzed (lactic, acetic and butyric acid), P1 silage had the highest percentage of lactic acid (2.78 ± 0.18%), which was significantly higher than P3 ($p<0.05$) but not P2 ($p>0.05$, Table 1). This heightened amount of lactic acid in P1 and P2 is also a result of the high carbohydrate content in *H. acutigluma* versus *N. oleracea Lour*, resulting in greater LAB levels and fermentation[13]. Furthermore, if the carbohydrate content found in forages used in silage is low, there will not be well ensilage because the production of other organic acids will be disrupted[14].

While lactic acid production is known to affect acetic acid formation during the ensilage process, the present results did not reveal a significant difference ($p>0.05$) in acetic acid content between any of the silage samples (Table 1). Moreover, the lower acetic acid versus lactic acid content in all three silage types suggests that there were fewer heterofermentative than homofermentative LAB species present during the ensilage process. Homofermentative LAB species, such as *Lactobacillus Plantarum*, *L. Brevis* and some *Pediococcus* sp., produce about 90% of the lactic acid found in forage silage, while heterofermentative species produce other products, such as acetic acid, ethanol and CO_2[15]. Nonetheless, the average percentage of acetic acid in all three silage types (1.00-1.02%) was well-above the standard percentage of acetic acid found in good silage (0.50-0.80%). This high acetic acid content is related to the amount of lactic acid produced in the silage[3,16]. If the amount of lactic acid produced is low and cannot sufficiently decrease the pH, a second fermentation process will occur by heterofermentative LAB which further degrades lactic acid into acetic acid, CO_2 and ethanol, thereby increasing the acidity (pH).

The amount of butyric acid produced was not significantly different ($p>0.05$)among any of the three silage types (Table 1). This is most likely due to alarge amount of lactic acid produced and relatively small amount of fungal growth present on the silage[17], which are both good indicators of high-quality silage. The standard percentage of butyric acid in good silage is about 0.10%[3] and good silage will be dominant in lactic acid[18]. Variations in the percentage of butyric acid present in silage are also influenced by the lactic acid content. A large amount of lactic acid will quickly decrease the pH of the silage, inhibiting butyric acid-producing bacterial growth, such as *Clostridium* sp.

Table 1: Lactic acid bacteria (LAB) content and organic acid profile of swamp forage silages

Treatments	LAB amount (Log CFU mL^{-1})	Total acid (%)	Lactic acid (%)	Acetic acid (%)	Butyric acid (%)
P1	8.24 ± 0.28^{b}	3.80 ± 0.67^{b}	$2.78\pm0..18^{b}$	1.00 ± 0.00	0.35 ± 0.09
P2	7.61 ± 0.41^{a}	3.06 ± 0.49^{a}	2.50 ± 0.42^{b}	1.00 ± 0.00	0.37 ± 0.05
P3	7.47 ± 0.40^{a}	2.60 ± 0.47^{a}	1.75 ± 0.34^{a}	1.02 ± 0.03	0.38 ± 0.07

[a,b,c]Different superscripts in the same column indicate a significant difference ($p<0.05$). Data presented as Means±standard deviations of five replicates. P1 (100% *Hymenachne acutigluma*), P2 (50%: 50% *H. acutigluma*: *Neptunia oleracea Loui*); P3 (100% *N. oleracea Loui*)

Table 2: Organic acid salt produced from 100% *Hymenachne acutigluma* silage liquid and its effect on bacteria drag zones

Base treatment	Organic acid salt produced (g)	pH (acidity)	*Escherichia coli* (cm)	*Salmonella typhimurium* (cm)
NaOH	38.993±1.052[a]	12.253±0.057[c]	0.113±0.025[a]	0.125±0.029[a]
$Ca(OH)_2$	85.458±2.937[b]	11.218±0.078[b]	0.163±0.048[a]	0.188±0.025[b]
ZnO	88.975±2.204[b]	7.438±0.101[a]	0.385±0.013[b]	0.213±0.025[b]

[a,b,c]Different superscripts in the same column indicate a significant difference ($p<0.05$). Data presented as Means±standard deviations of five replicates

Organic acid salt production from silage liquid and its effect on pathogenic bacteria drag zones: Organic acid salt formed as a result of adding a base [NaOH, $Ca(OH)_2$, or ZnO] to the acidic liquid. One major advantage of organic acid salts is their antibacterial potential. At this stage, the organic acid salts produced from P1 silage (100% *H. acutigluma*) were considered to be optimal based on the acid profile and high amount of LAB (Table 1). The organic acid salt obtained was tested against pathogenic bacteria *in vitro* to determine its inhibitory effect on growth. The effects of the addition of P1 organic acid salts on the growth of pathogenic bacteria can be seen in Table 2.

The pH of each organic acid salt liquid was significantly different ($p<0.05$). The most basic liquid resulted from the addition of NaOH (pH 12.253±0.057) and the most acidic by addition of ZnO (pH 7.438±0.101, Table 2). However, all organic acid salt samples were alkaline (basic) in pH. Reactions between weak acids and strong bases will produce alkaline salts due to the release of hydroxide ions[5,19] and stronger bases produce salt solutions with a higher pH[5]. Hence, NaOH (strong base) treatment produced the highest pH organic acid salt liquid in accordance with research conducted by Toledano *et al*.[20].

Interestingly, the total amount of organic acid salt produced by reacting silage liquid with NaOH was significantly lower ($p<0.05$) than that obtained by addition of $Ca(OH)_2$ and ZnO, which did not produce significantly different amounts between them ($p>0.05$, Table 2). This is likely related to the solubility of each base in water. When reacted, a strong base(NaOH) will dissolve more quickly in water than weaker bases [$Ca(OH)_2$ and ZnO]. Thus, during the course of the reaction, NaOH molecules had less time to form sediment and were wasted with the solution (supernatant), whereas molecules of $Ca(OH)_2$ and ZnO, which are slow to dissolve in water, will have more time to form sediment (salt)[5,21]. This can also explain the lower pH of the organic acid salt solution produced by the addition of $Ca(OH)_2$ and ZnO. However, it is possible that longer reaction time would greatly increase the amount of organic acid salt formed by NaOH since a high solubility would increase the number of reactant interactions, causing greater conversion to the crystalline product[22].

The organic acid salt solutions also showed significant effects on *in vitro* pathogenic bacteria (*Escherichia coli* and *Salmonella typhimurium*) growth, according to drag (inhibition) zone values (Table 2). Silage liquid reacted with ZnO produced the highest drag zone values (i.e., greatest growth inhibition) for both *E. coli* [0.385±0.013 cm, $p<0.05$ versus NaOH and $Ca(OH)_2$] and *S. typhimurium* (0.213±0.025 cm, $p<0.05$ versus NaOH), while NaOH produced the lowest (0.113±0.025 and 0.125±0.029 cm, respectively). The drag zone values of *E. coli* and *S. typhimurium* produced by extracts of ZnO organic acid salt are similar to previous reports which showed that organic acid salts produced by ZnO reaction with complete corn ration silage (Zn-C) produce the greatest inhibition against *E. coli* and *S. typhimurium* (0.33 and 0.34 cm)[23]. The organic acids inhibit growth by entering the cell membrane of pathogenic bacteria[24]. Organic acid salts of ZnO will dissociate the organic acid and Zn^{2+} ions in the digestive tract. Inactivation of bacteria by ZnO is due to the direct interaction between Zn^{2+} ions and the bacterial cell surface, which affects cell membrane permeability, allowing particle entry and subsequent induction of intracellular oxidative stress, thereby inhibiting bacterial cell growth[25].

In contrast, silage liquid reacted with NaOH had the least inhibitory power against both *E. coli* and *S. typhimurium* because endophytic bacteria can tolerate up to 10% Na^+, although their growth rates decrease with increasing Na^+ ion concentration[26]. The organic acid salts produced by $Ca(OH)_2$ did not optimally inhibit *E. coli* and *S. typhimurium* possibly because of the slow speed with which Ca^{2+}dissociates from organic acids. Previous study showed that use of $Ca(OH)_2$ for at least 1 week can inhibit 92.5% of bacterial growth[27]. Nevertheless, it seems that *S. typhimurium* is more resistant to all organic acid salt extract treatments compared to *E. coli*. Similar results were obtained by Sakaridis *et al*.[28] who reported that *Salmonella* sp. are more resistant to antimicrobials (penicillin, erythromycin, vancomycin and clindamycin) than *E. coli* and most of its biomass (>90%) has resistance to three other antimicrobial agents, such as tetracycline, oxytetracycline and streptomycin.

CONCLUSION

The present study revealed that *H. acutigluma* swamp grass silage (P1) had the best organic acid profile versus silages containing *N. oleracea Lour* (P2 and P3) due to a

higher amount of active LAB. Furthermore, the addition of 12.5% ZnO to P1 silage liquid produced the most organic acid salt with the lowest (most acidic) pH and showed optimal inhibition of both *E. coli* and *S. typhimurium* growth *in vitro*. These results suggest that Indonesian *Hymenachne* and *Neptunia* swamp forages and their silages may be good-quality, inexpensive and easily-cultivated alternative feed additives.

SIGNIFICANCE STATEMENT

This study reports the potential of swamp forage as a source of probiotics that could benefit poultry, especially for bacterial problems that interfere with the digestive system. This study will help researchers to uncover critical areas of swamp forage utilization that many researchers were not able to explore.

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