Lactic acid bacteria as microbial inoculant for *Acacia mangium* silage

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Abstract

Acacia mangium trees are widely distributed in Malaysia. It is a good protein source for animal feeding and contains about 14-17% dry matter of crude protein. However, this plant also has high amount of anti-nutritional factor, especially condensed tannins (more than 3% DM). This compound is able to bind to other macromolecules such as proteins and carbohydrates that lead to feed indigestibility in ruminants. The objectives of this experiment were to evaluate the effects of lactic acid bacteria inoculation on the reduction of condensed tannins in *Acacia mangium* silage, and their effects on the proximate and fibre composition of the silage. In this study, several species of lactic acid bacteria (LAB) with tannin-tolerant characteristics were isolated from rumen of Kedah-Kelantan cattle and were identified by molecular analysis. The tannin-degrading activity of the isolated LAB was determined between silage method and untreated silage. The amount of condensed tannin, proximate composition and fibre content were measured in percentage of dry matter (% DM) basis. A highly significant reduction ($p<0.05$) of condensed tannin in *A. mangium* silage was shown after inoculation with LAB. Approximately about 40% DM of condensed tannin was reduced with no significant reduction in protein and fat contents. The amount of NDF, hemicellulose and cellulose reduced ($P<0.05$) with the inoculation of LAB in the silage about 10.64%, 11.80%, and 29.59%, respectively. In conclusion, results demonstrate that the LAB inoculation used in this study has CT-degradation activity as well as cellulytic activity that is preferable to be used as microbial inoculation for tanniferous plant silage like *Acacia mangium.*

Keywords: Condensed tannins, lactic acid bacteria, *Acacia mangium*, silage, microbial inoculant.

Introduction

Acacia mangium Willd. is one of the most important forest plantation species found in Malaysia. The species was first introduced to Sabah in 1967 as a fire-break species. To date, more than 64 000 ha of A. mangium have been planted in Peninsular Malaysia for compensatory forest plantation, mainly in the state of Johore, Negeri Sembilan, Pahang, and Selangor. These plants are found to contain high crude protein (14-17%) which could be used as animal feed. However, it also contains high amount of tannins which were variable depending on the different ages, environmental conditions and different parts of the plant (Hoong et al., 2010).

Tannins are a complex group of water-soluble polyphenolic compounds arising from the metabolism of plants (Luciano et al., 2009). Tannins exist predominantly as hydrolysable tannins and condensed tannins. The major difference between the two types is the chemical structure where hydrolysable tannins have ester-linkage that is easily hydrolysed to produce small molecules while condensed tannins are much more resistant to biological and chemical degradation. Condensed tannins affect performance of

ruminants primarily through excessive protein binding. Effects of condensed tannins in ruminants vary with the type of tannin and plant sources, also different ruminant species vary in responses to condensed tannins (Min et al., 2003).

Lactic acid bacteria (LAB) have widespread use in fermented food production (Hoque et al., 2001) and are generally recognized as safe (GRAS) organisms and can be safely used for medical and veterinary applications (Fuller et al., 1989). In the ruminant industry, feed cost can contribute up to 70% of total production cost, therefore improving the low quality and palatability of feed can have a significant impact on the profitability of a ruminant enterprise.

Improving the nutrient utilization and the available energy from livestock feeds has been investigated over the years. In high forage production systems, many of the current processing methods are less efficient at increasing energy content and alternative methods have then been used for this purpose. Forage digestibility is the major factor affecting the intake of available energy for ruminants, even with the improvements in forage breeding and agronomic practices (Huang et al, 2011).

Producing high-quality forage as silage, while avoiding DM losses as much as possible, is a challenge. Dry matter losses and quality changes occur during each of these stages of the ensiling process, reducing the quality of the as fed product. As the silo becomes anaerobic, various anaerobic and facultative microorganisms increase in population and ferment primarily sugars and organic acids in the crop. The principal fermentative microbial groups include LAB, enterobacteria, clostridia, and yeasts (Borreani et al., 2018). The losses associated with the fermentation in the silo are primarily from carbon dioxide production. These losses typically are in the range of 2 to 4%.

Without the use of silage additives, the fermentation process is a result of the activity of the epiphytic microorganisms on the crop being ensiled. The populations of various microbial groups on crops at ensiling are influenced by the crop, growing conditions, environmental factors during wilting, (Borreani et al., 2018; Muck et al., 2003). Of the bacterial silage inoculants, homofermentative LAB should be the most effective at minimizing carbon dioxide losses during the initial ensiling fermentation. The Lab inoculants have been developed to rapidly grow and lower pH in silage so they dominate fermentation (Kung et al., 2003). Overall, inoculants are additives that can be used to improve silage quality when used appropriately together with good silage management (Muck, 2010). Therefore, the objective of this study is to use lactic acid bacteria as the silage inoculant to reduce the amount of condensed tannin in *Acacia mangium* silage.

Materials and Methods

Lactic acid bacteria isolation

Lactic acid bacteria (LAB) used in this study were isolated from fistulated Kedah-Kelantan cattle at MARDI Kemaman, Terengganu, Malaysia. The animals have been adapted to acacia silage (untreated) for 7 d before the samples of rumen content were collected. The samples were kept under anaerobic condition and stored at 4°C prior to the start of the experiment. The rumen samples were spread on MRS agar and incubated at 37°C under anaerobic condition, and sub-cultured for several times until single colonies were obtained. Each colony was individually cultured into fresh MRS broth for 24 h and the bacterial isolates were identified by molecular analysis using 16sRNA sequencing. The 16sRNA primers

used in this study were 785F: GGATTAGATACCCTGGTA and 907R:CCGTCAATTCMTTTRAGTTT. The LAB used are listed in Table 1. The capability of the isolates to grow in condensed tannin (CT) was studied in MRS broth-containing CT extracts (10% w/v).

Condensed tannin degradation by LAB

Each of bacterial isolates was cultured in CT-containing medium to evaluate their ability to degrade these compounds by comparing the percentage of tannin reduction. The experiment was conducted in triplicates. The amount of CT was assayed by Vanillin-HCl assay following the method described by Makkar et al. (1987).

Silage preparation

The tannin degrading activity of the LAB isolates in *A. mangium* was determined by silage method. Whole acacia plants with height of 1 to 1.5 m were harvested and chopped to 2-3 cm length. The following treatments were applied to the fresh forage: 1) Control (untreated), and 2) LAB ($1x10^5$ cfu/g fresh acacia. An amount of chopped acacia (300 g) from each treatment was packed into 500 mL glass jar in triplicates, sealed with lid and adhesive tape and stored at ambient temperature (average 25°C).

Proximate composition

After the fermentation period, the silos were opened and homogenised and sampled to determine the DM content, proximate composition, fibre and tannin content. Percentage of DM was measured based on dry weight of sample over fresh sample weight in percent; by drying samples at 105°C overnight. The proximate and fibre composition analyses were conducted following the method by Gul and Safdar (2009).

Tannin extraction and analysis

Condensed tannins were extracted from the *A. mangium* silage using aqueous acetone/diethyl ether as described by Terrill et al. (1992). The CT were extracted from 2 g of dried samples in 50 ml extraction solvent (70% (v/v) aqueous acetone containing 0.1% (w/v) ascorbic acid. Aqueous acetone was the best solvent for extraction of tannin when compared to water, aqueous methanol and the order of efficiency was acetone > methanol > water (Rama and Prasad, 1991). Diethyl ether was used to remove chlorophyll, pigments and low molecular weight phenolic acids. Traces of acetone and diethyl ether in the extracts were further evaporated under vacuum in a rotary evaporator at 40°C and the extracts were lyophilized at -56°C for 48 h. The yield extracts were measured. The concentration of condensed tannin in each fraction was measured using modified vanillin-HCl assay (Martin, 1978; Tohru, 1998). Catechin and vanillin were purchased from Sigma Aldrich, USA and all other reagents were of analytical grade. A 20µL of fractions were seeded into 96-well plate and 120 µL of Vanillin reagent and 60 µL of concentrated HCl were added into each well. The plate was incubated for 15 min in the dark and absorbance was recorded at wavelength of 500 nm using an Epoch, BioTek microplate reader. The amount of CT in plant extracts were measured in values of concentration before converted to percentage values. Catechin was used as a standard measurement at range of 25 to 300 µg/mL. The standard curve generated was based on the absorbance at 500 nm versus concentration of catechin with

the standard equation was y=0.0021x. The calculated percentage of condensed tannin in *A. mangium* are presented in Table 2.

Statistical analysis

The experimental design was completely randomized treatments with three replicates. The data were subjected to ANOVA using SAS software Version 9.4 (Statistical Analysis System, 1991). All the determinations were carried out in triplicates and data were expressed as mean ± standard deviation. The means were separated by Tukey's test, and the significance level was $P<0.05$.

Results and Discussion

Inoculants containing principally LAB are commonly used as silage additives in order to improve preservation efficiency. However, in this study, the LAB were added to *Acacia mangium* silage to study their ability to reduce condensed tannin in the plant. There were six homofermentive bacterial species isolated from rumen of Kedah-Kelantan cattle. They were *Weissella ciberia, Pediococcus acidilactici, Lactobacillus fermentum, Enteroccocus hirae, Lactobacillus plantarum* and *Enterococcus viikkiensis* (Table 1). They are called homofermenters because only lactic acid is produced in the growth culture medium, as reported by Muck and Kung (1997), except for *Weissella cibaria*. Lactic acid was produced by all strains at range of 2 to 7 g/L with various levels of L-type and D-type lactic acid. *Enterococcus viikkiensis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Weissella cibaria* produce more D-lactic acid than L-lactic acid, whereas *Enterococcus hirae* produce only L-lactic acid with trace amount of D-lactic acid.

The final pH of each isolate culture and percentage of CT reduction in 1% of *A. mangium* CT extract are presented in Table 1. It shows an acidic environment was produced in the growth medium at pH range between 3.57 to 4.37. It can be explained by the production of lactic acid in the growth medium. Each of bacterial strains is also able to reduce the amount of condensed tannin with *Weissella cibaria* giving the highest reduction, followed by *Enterococcus hirae*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Pediococcus acidilactici* and the least reduction was found in *Enterococcus viikiensis*.

Table 1. Lactic acid bacterial species from rumen of Kedah-Kelantan cattle and their characteristics in lactic acid production and condensed tannins reduction

Bacterial isolates	Types of Lactic acid		Total Lactic acid (g/L)	CT reduction (%)	pH
	L- (g/L)	D- (g/L)			
Enterococcus hirae	2.73	0.02	2.75	30.56	4.37
Enterococcus viikiensis	1.91	3.18	5.08	26.11	4.11
Lactobacillus fermentum	1.91	4.11	6.02	29.26	4.22
Lactobacillus plantarum	2.95	4.08	7.03	30.40	3.57
Pediococcus acidilactici	2.44	3.67	6.11	28.93	4.27
Weissella cibaria	2.50	3.51	6.00	35.40	4.32

The findings from proximate composition of *A. mangium* silage are presented in Table 2. There are no significant difference in energy content between fresh sample, untreated silage and LAB-treated silage, where the ME values were 9.49 MJ/kg, 9.21 MJ/kg, and 9.45 MJ/kg, respectively. The fibre content also shows a variation in values in LAB-treated silage. The percentage of NDF, hemicellulose and cellulose were significantly reduced compared to fresh sample which showed fibre degradation properties of the LAB inoculant. However, the percentages of total crude fibre, ADF and lignin indicated no difference. The CP and crude fat of LAB-treated silage were not significantly different with fresh sample, indicating the LAB treatment could maintain the amount of protein and lipid in the plant samples even after ensiling process. The ash content showed a slightly lower value after the treatment with LAB.

Table 2. Nutritional values and condensed tannins content of *Acacia mangium* after the treatment with lactic acid bacteria, comparison with fresh and untreated samples

Nutritional values	Acacia (Fresh)	Acacia silage (Untreated)	LAB-treated Acacia silage
Energy			
TDN (%)	63.03 ± 1.27	61.37 ± 1.21	62.82 ± 0.85
ME (Mj/kg)	9.49 ± 0.21	9.21 ± 0.20	9.45 ± 0.14
Fibre			
ADF (%)	38.65 ± 1.16^{a}	42.85 ± 0.10^{b}	39.41 ± 0.64^{a}
NDF (%)	64.37 ± 2.14^{a}	59.62 ± 1.49^{b}	57.52 ± 1.11^{b}
Lignin (%)	26.94 ± 2.49^{a}	28.84 ± 1.41^{b}	23.76 ± 1.72^{b}
Hemicellulose (%)	25.72 ± 1.53^{a}	16.77 ± 1.83^{ab}	18.11 ± 0.68^{b}
Cellulose (%)	11.72 ± 1.35^{ab}	14.01 ± 1.93^{b}	15.66 ± 0.05^{a}
CF (%)	27.10 ± 1.40^{a}	30.07 ± 0.69^{b}	28.87 ± 0.69^{ab}
Proximate composition			
Ash (%)	4.64 ± 0.24^{a}	3.61 ± 0.07^{b}	3.92 ± 0.16^{b}
CP (%)	17.09 ± 0.70^{a}	15.53 ± 0.20^{b}	16.70 ± 0.12^{a}
Dry matter (%)	36.31 ± 2.58^{a}	32.18 ± 0.70^{b}	32.22 ± 0.56^{b}
Crude fat (%)	2.47 ± 0.28	2.75 ± 0.92	3.34 ± 0.63
NFE (%)	48.69 ± 1.17	48.04 ± 0.63	47.17 ± 1.14
Condensed tannin (%)	3.25 ± 0.27^{a}	2.45 ± 0.20^{a}	1.94 ± 0.09^{b}

^{ab}Means with different superscript in the same rows differ significantly ($P<0.05$)

In this experiment, there were significant differences observed in percentage of CT (1.94%) in LAB-treated silage compared with untreated-silage (2.45%) and fresh sample (3.25%) (Table 2). Wina et. al. (2010) also reported the amount of condensed tannin in *Acacia mangium* bark was determined as 3.44%. It was also found that the amount of

condensed tannin reduced to about 24.6% by the silage fermentation itself compared to fresh Acacia sample. The inoculation of LAB in acacia silage reduce the amount of condensed tannin by 40%, whereas the amount of crude protein was found to have minimum reduction, compared to the other treatment and control group. Condensed tannins reduced protein degradation in the rumen and increase the flow of amino acids to the intestine for absorption. Of greatest importance is their ability to reduce the breakdown of plant proteins in the rumen so that more amino acids reach the intestine (Waghorn and Shelton, 1998). Therefore, it is preferable to only reduce the amount of condensed tannin in the feed compared to eliminate them all.

The amount of NDF, hemicellulose and cellulose reduced ($P<0.05$) with the inoculation of LAB in the silage about 10.64%, 11.80%, and 29.59%, respectively. The reductions in condensed tannin and cellulose content seen in this study therefore may suggest that LAB used in this study has the CT-degradation and cellulolytic activities. From other study, Niezen et. al. (1998) suggested to use polyethylene glycol (PEG) to remove the effects of CT which could to be used as an alternative method to feed forage containing CT. Many analytical methods have been used to quantify tannins in plant materials. Commonly used methods include oxidative depolymerization of CT reactions of the A ring with an aromatic aldehyde, and oxidation–reduction reactions (Waterman and Mole, 1994). Other methods involve acid cleavage reactions, precipitation reactions, enzyme and microbial inhibition and gravimetric procedures (Schofild and Mbugua, 2001). However, vanillin-HCl method is the simplest method to assay the amount of condensed tannin in the plant samples by using catechin as the standard.

Since the silage method is commonly used to preserve feed by fermentation process, the inoculation of bacteria into the silage may also react on and degrade the condensed tannin contained in the plant sample. Therefore, the selection of correct strains is crucial with the ability of them to maintain the quality of the feed in the silage and also improve the nutrient quality by reducing the antinutritional factors present in the feed component.

Conclusion

On the basis of the comparative assessment, lactic acid bacteria isolated from local Kedah-Kelantan cattle were able to reduce condensed tannins in *Acacia mangium* and able to maintain protein and energy content in Acasia silage. The overall interpretation of the present investigation may offer a scientific basis for increased and versatile utilization of these lactic acid bacteria as a silage inoculant for high tannin plants and low quality forages.

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