P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com
Research Article https://doi.org/10.47278/journal.ijvs/2023.021

Pre-Treatments of *Sonneratia Alba* Fruit as The Potential Feed for Ruminants Using *Aspergillus Niger* at Different Fermentation Times: Tannin Concentration, Enzyme Activity, and Total Colonies

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Article History: 23-140 Received: 08-Feb-23 Revised: 27-Feb-23 Accepted: 07-Mar-23

ABSTRACT

The aim of this study was to reduce high level of tannin in mangrove (*Sonneratia alba*) fruits by fermentation using *Aspergillus niger*. A completely randomized design was used with four treatments (fermentation time) i.e. A: 7 days, B: 10 days, C: 13 days, D: 16 days and the experiments were performed in four replicates. The parameters measured were total mold colony, tannase enzyme activity, tannin content, and tannin degradation. The results showed that the fermentation time had a significant effect ($P<0.05$) on total mold colony, tannase enzyme activity, tannin content, and tannin degradation. The highest total colony and tannase enzyme activity were found in treatment B, namely 2.24 x 10^{14}cfu/mL and 7.465U/mL, while the lowest tannin content and highest tannin degradation were found in the treatment D, namely 16.03% and 24.42%. The conclusion of this study was that on sixteenth days (Treatment D) fermented *Sonneratia alba* fruits produced the lowest levels of tannin (16.03%) with the highest level of degradation (24.42%).

Key words: *Aspergillus Niger*, Fermentation Time, Ruminant Feed, *Sonneratia Alba*, Tannin.

INTRODUCTION

Mangrove is a potential plant as an alternative feed for ruminants (Sari et al. 2021; Yanti et al. 2021; Sari et al. 2022a, 2022b, 2022c). *Sonneratia alba* is a mangrove plant found in coastal areas (Wintah et al. 2021). This plant grows in confluence areas between rivers and estuaries or muddy bays that have total litterfall production about 79.19gm/m^2 in 28 days (Rusianti et al. 2022). *S. alba* trees can produce fruit in two fruiting periods: April to June and September to November (Sahromi 2011). *S. alba* fruit is very feasible to be developed. In a relatively short time, *S. alba* tree can produce 2kg of fruit per day. Although this plant has a lot of yields, it has not been widely used. (Jariyah and Nurismanto 2017; Tahir et al. 2023).

Sonneratia alba fruit is not poisonous, so it can be eaten immediately. The benefits of *S. alba* fruit are the sour fruit can be used to make vinegar or syrup, produces pectin, has antioxidant activity, anti-*Escherichia coli*, effective for increasing appetite, and breath root as a substitute for cork and floaters (Wintah et al. 2021; Pakadang et al. 2021; Riyadi et al. 2022). Apart from being used as a food ingredient, *S. alba* fruit has the potential to be used as a feed ingredient for ruminants because it contains flavonoid compounds that can enhance rumen microbial activity. Wonggo et al. (2017) stated that the methanol extract of *S. alba* fruit taken from the village of Wori, North Sulawesi, Indonesia, contained bioactive compounds such as phenolics, flavonoids, steroids, triterpenoids, saponins, and tannins. The results of nutritional contents of young *S. alba* fruit were 89.47% dry matter, 94.82% organic matter, 5.18% ash, 8.74% protein, 1.44% fat, and 74.12% carbohydrates. The proximate content of old *S. alba* fruit was 90.37% dry matter, 94.61% organic matter, 5.39% ash, 8.34% protein,

Cite This Article as: Elihasridas, Pazla R, Jamarun N, Yanti G, Sari RWW and Ikhlas Z, 2023. Pre-treatments of *Sonneratia alba* fruit as the potential feed for ruminants using *Aspergillus niger* at different fermentation times: Tannin concentration, enzyme activity, and total colonies. International Journal of Veterinary Science 12(5): 755-761. https://doi.org/10.47278/journal.ijvs/2023.021

1.54% fat, and 75.1% carbohydrates (Ardiansyah et al. 2020). The tannin contents of *S. alba* is quite high, the fruit contains 41.6% tannin, the leaves contain 29.12% tannin, and the bark contains 4.16% tannin (Bay 2016).

Tannins are anti-nutritional polyphenol compounds found in many impactful plants and it can have a negative effect on livestock nutrition (Popova dan Mihaylova 2019; Verma et al. 2021). Tannins are polyphenolic compounds in plants as anti-nutrients (Jamarun et al. 2020). Tannins generally consist of two types, namely condensed tannins and hydrolyzed tannins. Condensed tannins and hydrolyzed tannins are found in plants, but condensed tannins are more dominant in plants. Condensed tannins are polymers of flavan-3-ol found in plants (Nath et al. 2022). Condensed tannins have a high level of stability, are more difficult for enzymes to digest, and are used as bypass nutrients. In contrast, hydrolyzed tannins have a low level of stability, so they are easily broken down into phenolic groups and simple sugars (Hidayah 2016). Rira et al. (2022) reported that condensed tannins could bind proteins in the rumen and reduce protein degradation.

The tannin content in feed ingredients can be reduced in various ways, such as soaking, boiling, and fermentation (Jamarun et al. 2021). Ikhlas et al. (2023) stated that the use of lime water with a concentration of 5% for 10 minutes can reduce tannins up to 4.54%. Fermentation is a technology to improve the quality of animal feed using microorganisms (Ciptaan et al. 2022; Syamsuddin et al. 2022). Mold is the most commonly used microorganism for tannase production (tannin acyl hydrolase). *Aspergillus niger* have ability to produce tannase enzymes and it is often used to reduce tannin content (Anwar et al. 2009; Espitia et al. 2022). Tannase enzymes are a class of compounds that can change hydrolyzed tannin ester bonds between glucose and esters. According to Kumar et al. (2013), this enzyme can reduces the tannin content. Research conducted by Pakaweerachat et al. (2022) also reported that *A. niger* had the best ability to produce tannase enzymes. The food industry uses tannase enzyme in instant tea products, beer and fruit juices (Banerjee et al. 2001). Tannase is also used to remove unwanted properties from fruit juices (Kanpiengjai et al. 2020). In recent years, various research groups have been involved in efforts to improve simple methods for the biosynthesis and purification of tannases on a small scale. In addition, major focus is needed on enzyme regulation, design of new bioprocess systems, new expression systems, new development strategies for large-scale processes using tannin-rich agro-industrial experimental wastes, new downstream processing for industrial applications and simple design of new applications for tannase (Lekshmi et al. 2021).

The fermentation process is strongly influenced by dose and time factors. The dose level is related to the size of microbial population, which has the opportunity to determine the speed of microbial development in producing enzymes to break down substrates, which in turn will affect the final product (Pazla et al. 2020; Mirnawati et al. 2022). Microbial growth is characterized by the length of time it is used so that the metabolic concentration increases until it finally becomes limited, which then can leadto the decrease of growth rate (Pazla et al. 2021a). This study aimed to determine the quality of *S. alba* fruit as ruminant feed fermented with *A. niger* at different fermentation times regarding tannin content, enzyme activity, and total mold colonies.

MATERIALS AND METHODS

Ethical Approval

This study did not need ethical approval as it was not related to animals.

Experimental Site

This research was conducted in the feed technology laboratory, Faculty of Animal Husbandry, Andalas University. *S. alba* fruit samples were taken from Pasaman mangrove forest, West Sumatra, Indonesia.

Rejuvenation of *A. Niger*

About 50mL of PDA mushroom growth media was prepared and being sterilized in autoclave for 30min at 12°C. The media was then compacted and *A. niger* was inoculated into each test tube, then incubated for seven days.

Production of *A. Niger* Inoculum

As much as 100gm of bran was weighed and added with water to 60% water content, and then being homogenized in heat-resistant plastic. It was then autoclaved for 30min to sterilize. After sterilization, it was cooled in temperature of 35-37ºC, and about 5mL of Brook's solution was added, then 1 test tube of oblique media containing *A. niger* was inserted. It was then being incubated for seven days, after that the inoculum was ready to be used.

Sonneratia Alba Fruit Fermentation by *A. Niger*

Sonneratia alba fruit was chopped and then weighed fresh. Samples were baked at 55ºC for 24hrs. Then the samples were mashed to become flour. About 100gm of each material for all treatments were weighed and added with water until it reaches a moisture content of 60%, and 6% of the *A. niger* inoculum was then added. It was then homogenized, flatten to equal thickness and incubated for 7, 10, 13, and 16 days. Samples in each treatment were set aside 15gm fresh for testing enzyme activity and total colonies. Samples were dried at 80ºC for 4hrs and lowered to 60ºC for 20hrs. The fermented product was ready to be tested for tannin content. A standard solution of 225µg/mL tannic acid was pipetted into each 1.0mL; 2.0mL; 4.0mL; 5.0mL; and 6.0mL into a 25mL volumetric flask. Each of them was added with 3.0mL of iron (III) ammonium disulfate, stirred for 20min, added 3mL of potassium iron (III) cyanide, stirred for 20min, then added aqua demineralization to obtain a series of solutions 1; 2; 4; 5 and 6µg/mL. The absorbance was measured at 720nm. A total of 5mL of fermented sample extract was added with aqua demineralization to a volume of 10mL, then 1mL was taken and put into a 25mL volumetric flask and added with 3.0mL of iron (III) ammonium disulfate, stirred for 20min, added 3mL of iron potassium (III) cyanide, stirred for 20min. About 25mL of distilled water was added, and the absorbance was measured at 720nm.

Tannase Enzyme Activity

Enzyme activity was determined based on the method of Bajpai dan Patil (1997). A standard curve was made with added 100µl of the standard solution to 150µl of 0.667% (w/v) rhodanine solution and vortexed until homogeneous. About 2.25mL of 0.5N KOH solution was then added until the solution color was red. The absorbance was measured at 520nm. Plot absorbance values with gallic acid levels.

The enzyme activity of the sample (crude enzyme filtrate) was measured. Sample was centrifuged for 10min at 13000rpm. The filtrate obtained was hydrolyzed with a solution of 2.5mL of 2N H_2SO_4 for every 0.5mL of crude enzyme. The mixed enzyme and acid solution was heated for 26hours at 100^{o}C (performed in a water bath). After hydrolysis, 100µL was taken, and 150µL of rhodanine solution was added and vortexed. After that, about 2.25 of 0.5N KOH solution was added and the absorbance was measured.

Research Design

This research used a completely randomized design with four treatments (fermentation time) and four replication each, so there were 16 research units. The treatments A, B, C, and D were given for 7, 10, 13 and 16 days, respectively. The mathematical model of the design is by design according to Steel and Torrie (1991).

$Yij = \mu + \tau i + \beta j + \sum ij$

Information:

Yij = the results of the observations of the i-th treatment and the j-th replication;

µ = general mean;

τi = the effect of treatment I;

∑ij =random error;

βj = the effect of the treatment j;

i = treatments (A, B, C, D);

j = repetition (1, 2, 3, 4)

Parameter Measured

Parameters measured in this study were tannin content, tannin degradation, total mold colonies, and tannase enzyme activity.

Statistical Analysis

Research data were analyzed using analysis of variance (ANOVA). Differences between treatments were tested using Duncan's Multiple Range Test using SPSS software (IBM SPSS Statistics, USA) version 21.0.

RESULTS

Total Colonies of Mold *A. Niger*

Fermentation time had a significant effect (P<0.05) on the total colonies of *A. Niger*. The highest total mold colonies were found in treatment B, which was ten days of fermentation, and the lowest one was found in treatment D, which was 16 days of fermentation with values of $2.24x10^{14}$U/mL and $1.4x10^{14}$U/mL respectively. The Effect of *S. alba* mangrove fruit fermentation time on total colonies using *A. niger* is presented in Fig. 1.

Tanase Enzyme Activity from *A. niger*

Fermentation time had a significant effect (P<0.05) on the tannase enzyme activity of *A. niger*. Tannase enzyme activity decreased with the length of fermentation time. The highest fungal tannase enzyme activity was found in treatment B, which was ten days of fermentation, and the lowest one was found in treatment D, which was 16 days of fermentation, with values of 7.465 and 5.953 U/mL, respectively. The Effect of *S. alba* mangrove fruit fermentation time using *A. niger* on tannase enzyme activity was presented in Fig. 2. The relationship between total mold colonies and tannase enzyme activity was presented in Fig. 3.

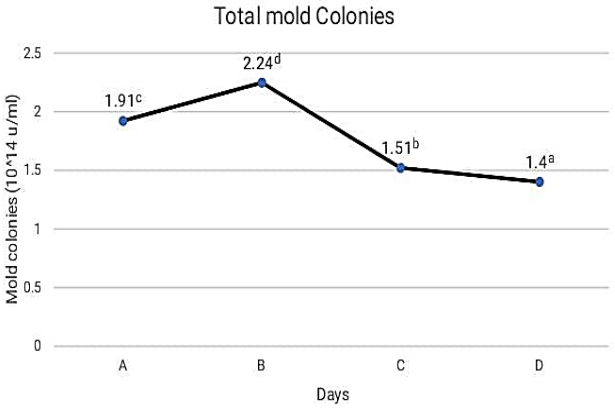

Fig. 1: Total mold colonies of fermented *S. alba* fruits by *A. niger* at different fermentation times. Treatments are at days 7 (A), 10 (B), 13 (C) and 16 (D).

Tannin Content and Tannin Degradation

Fermentation time had a significant effect (P<0.05) on the tannin content of the *S. alba* fruit. The tannin content decreased from 7 days to 16 days of fermentation, as shown in Fig. 4. The lowest tannin content was found in treatment D, 16 days of fermentation, with a value of 16.03%.

Fermentation time significantly affected tannin degradation (P<0.05). The tannin degradation value continued to increase with the length of the fermentation time, as shown in Table 1. The lowest tannin degradation value was found in treatment A (7 days), 8.72%, and the highest one was found in treatment D (16 days), 24.42%.

Table 1: Tanin degradation of fermented *S. alba* fruits by *A. niger* at different fermentation times

Treatments	Tanin Content (%)		Tanin degradation (%)
	Before Fermentation	After Fermentation	
A	21.21±0.61	19.36±0.43c	8.72±1.03d
B	21.21±0.61	18.30±0.51bc	13.72±2.11c
C	21.21±0.61	17.25±0.34ab	18.67±2.45b
D	21.21±0.61	16.03±0.31a	24.42±2.21a

Mean±SD in a column with different letters differ significantly (P<0.05). Treatments at days 7 (A), 10 (B), 13 (C) and 16 (D).

DISCUSSION

Total Colonies of Mold *A. Niger*

The increase of *A. niger* total colonies was related to the exponential phase, in which the fungi divided cells by utilizing the available substrate in the form of sugars and nutrients available in the substrate.

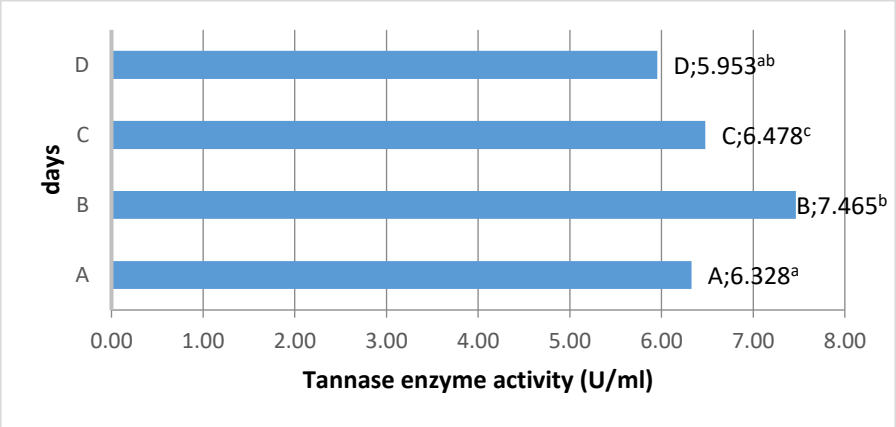

Fig. 2: Tannase enzyme activity of fermented *S. alba* fruits by *A. niger* at different fermentation times. Treatments are at days 7 (A), 10 (B), 13 (C) and 16 (D).

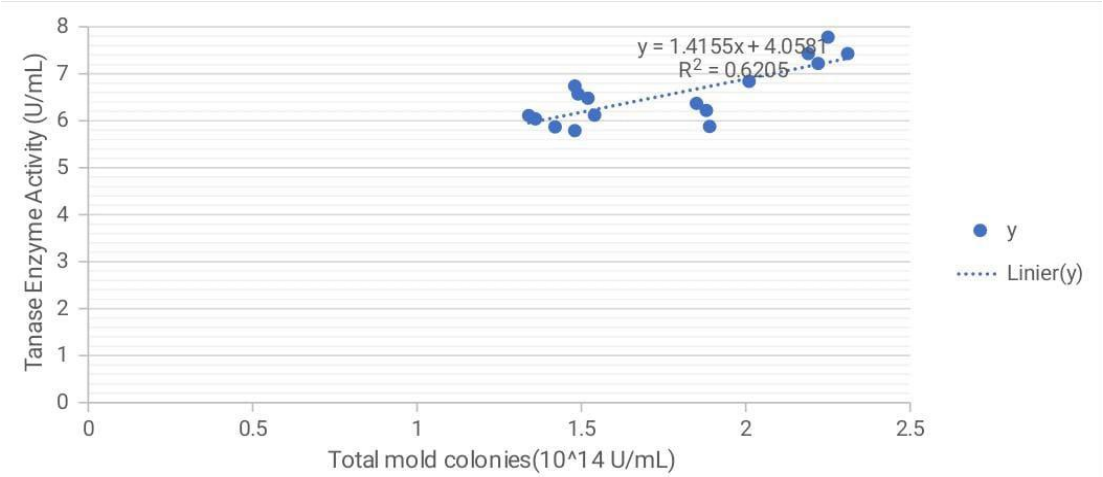

Fig. 3: Relationship between total mold colonies and tannase enzyme activity of fermented *S. alba* fruits by *A. niger* at different fermentation times. Treatments are at days 7 (A), 10 (B), 13 (C) and 16 (D).

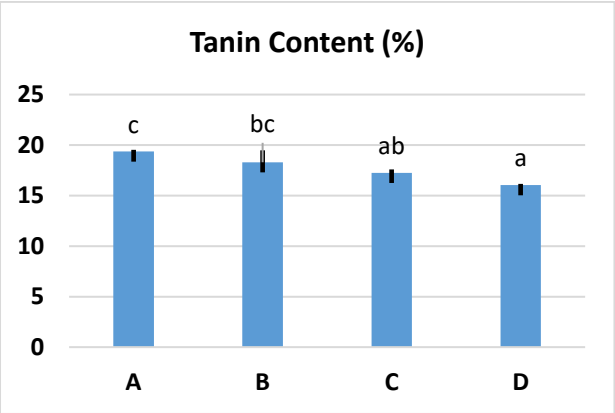

Fig. 4: Tannin content of fermented *S. alba* fruits by *A. niger* at different fermentation times. Treatments are at days 7 (A), 10 (B), 13 (C) and 16 (D).

Fermentation time significantly affected total mold colonies ($P<0.05$), with an increase in total mold colonies from 7 to 10 days of fermentation. The mold population began to decrease on the 10th to 13th day of fermentation. This condition occurred because the amount of available food was equal to the number of existing mold cells, so that cell multiplication did not occur. If this condition continues, the mold will experience a stationary/death phase because there will be a decrease in pH and an increase in CO_2 gas production, which inhibits the mold growth. Pazla et al. (2021b) stated that microbial growth is characterized by the time it is used so that the metabolic concentration increases until it finally becomes limited, which can lead to the decrease of growth rate. The number of mold colonies decreased drastically on the 16th day of fermentation. This was influenced by several factors such as unstable temperature, nutrients, and environmental conditions. In addition, it was also influenced by the entry of air from outside into a quiet place, allowing unwanted bacteria to mix between the two (Haryani and Hidayat 2012).

This follows the opinion of Bahri et al. (2019) who stated that inhibitor agents for mold growth include a pH that is too acidic or too alkaline and over concentration of CO_2 gas will lead to the unoptimal growth of the mold. Mold is a type of aerobic microbe that requires oxygen to support its life.

Tannase Enzyme Activity of *A. niger*

Figure 2 showed that the duration of fermentation had a significant effect ($P<0.05$) on the activity of *A. niger* tannase enzyme. The highest tannase enzyme activity was found in treatment B, which was 7.465U/mL at 10 days of fermentation. The activity of this enzyme continued to

decrease on the 13th and 16th day of fermentation, namely 6.478 and 5.953 U/mL, respectively. Enzyme activity increased only from 7 days to 10 days of fermentation. On the 16th day, the activity of the tannase enzyme was at its lowest value.

The activity of this enzyme is in line with the growth of mold colonies. The more colonies of mold, the higher enzyme activity produced. Treatment B, 10 days of fermentation, produced the highest total colonies and tannase enzyme activity. The decrease tannase enzyme activity on day 16 was due to the reduced number of molds because the molds were already experiencing a death phase. There is a strong correlation between tannase enzyme activity and total mold colonies, as shown in Fig. 3.

The results of linear regression calculations between total mold colonies and tannase enzyme activity obtained the equation: y = 1.4155x + 4.0581 (Fig. 3). Variance analysis showed a linear regression relationship between total mold colonies and tannase enzyme activity. The correlation coefficient (r) of this equation was 0.79 (P>0.05), which according to Sugiyono (2007), indicates that there is a strong relationship between total mold colonies and tannase enzyme activity. The coefficient determination (R^2) of this equation was 0.620, which means that 62% of the tannase enzyme activity was affected by total mold colonies. In comparison, 28% was influenced by other factors such as pH and temperature. The relationship between total mold colonies and tannase enzyme activity (Fig. 3) showed that high mold colonies will lead to the increase of tannase enzyme activity. There was a relationship between the total number of mold colonies and the activity of the tannase enzyme because the mold produces tannase enzyme during the fermentation process.

Tannin Content and Tannin Degradation

The results of tannin content of fermented *S. alba* fruit with *A. niger* were presented in Fig. 3. Fermentation with *A. niger* reduced the tannin content from 19.35% (Treatment A) to 16.02% (Treatment D). The value of tannin degradation continued to increase with the length of fermentation time (Table 1). Bhat et al. (1997) and Sherief et al. (2011) stated that the longer fermentation process will increase tannin degradation due to tannase enzyme activity produced by the mold. The ability of *A. niger* to hydrolyze tannins by the tannase enzyme is influenced by pH, tannin content, and fermentation time (Tanash et al. 2011; Tanash et al. 2012). Research conducted by Chauhan et al. (2022) showed that the longer fermentation will lead to the lower tannin content. The decomposition of tannins causes a decrease in tannin content into gallic acid and glucose during fermentation (Preethi and Kalpanadevi 2022).

The tannase enzyme works specifically to degrade tannins, breaking off ester-tannin bonds to form gallic acid and glucose. Tannase hydrolyzes ester bonds in galotanin, ellagitanin, complex tannins, and gallic acid esters. Tannase catalyzes the hydrolysis of tannic acid (nonagalloyl glucose) into gallic acid and glucose through tetragalloyl glucose and two types of mono-galloyl glucose (Chávez et al. 2018). The fermentation process requires varying lengths of time to produce the desired product. The length of fermentation affects the activity of microorganisms because microorganisms experience several growth phases. The first is the lag phase, or the phase of cell adaptation to the environment. Then the exponential phase is the phase of increasing the number of cells and cell activity. The three stationary phases is the phase of secondary metabolites can be harvested (Idiawati et al. 2015). Fermentation time can affect the secretion of tannase enzymes, which will reduce tannin levels in mangrove fruit. Research by Shi et al. (2005) showed that tannin degradation in unfermented valonea (*Quercus aegilops*) by *A. nigger* was 14.3% in 9 days. So, fermentation can reduce tannin levels.

Conclusion

The conclusion of this study was that on the sixteenth day (Treatment D) fermented *Sonneratia alba* fruit with *Aspergillus niger* produced the lowest tannin content (16.03%) with the highest degradation rate (24.42%).

Author's Contribution

Novirman Jamarun designed the concept, Elihasridas supervised the research, and Roni Pazla analyzed the data and wrote the draft. Gusri Yanti, Rani Winardi Wulan Sari, and Zaitul Ikhlas worked in the laboratory.

Competing Interest

All the authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank the research and community service institute Andalas University (LPPM UNAND), Indonesia who has funded this research (indexed publication research) with the contract number: T/62/UN.16.17/PT.01.03/Pangan-RPT/2022.

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