BIODIVERSITAS
Volume 24, Number 11, November 2023
Pages: 5863-5872

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241104

Fermentation of rain tree (*Samanea saman*) seed meal using mixed microbes to improve its nutritional quality

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Manuscript received: 4 September 2023. Revision accepted: 9 November 2023.

Abstract. *Anwar A, Zainuddin, Djawad MI, Aslamyah S. 2023. Fermentation of rain tree (*Samanea saman*) seed meal using mixed microbes to improve its nutritional quality. Biodiversitas 24: 5863-5872.* Rain tree (*Samanea saman*) seed meal is a source of protein; however, its utilization remains limited due to the presence of anti-nutrients, such as tannins acting as protein inhibitors, high crude fiber content, dissolved protein, and low digestibility of dry and organic matter. Fermentation using mixed microbes potentially enhances the nutritional value of rain tree seed meal. This study aims to improve the nutritional quality and reduce anti-nutritional factors in rain tree seed flour using mixed microbes at various doses and incubation times in vitro. Microbes utilized in this study include *Bacillus* sp., *Saccharomyces cerevisiae*, and *Rhizopus* sp. The study was designed using a Factorial Completely Randomized Design, using two factors, i.e., 3 doses of mixed microbes (0, 1.5, 3, and 4.5 mL/100 g rain tree seed meal) and 3 different incubation times (42, 72 and 96 hours). There were significant interactions between the microbe doses and incubation times. The treatment of 4.5 mL of mixed microbes/100 g rain tree seed meal and a 72 hours incubation time reduced substantially crude fiber content (59.60%) and crude fat (73.20%), coupled with an increase in crude protein content (11.62%), NFE (6.52%), dry matter digestibility (DMD) (36.78%), organic matter digestibility (OMD) (50.42%), and dissolved protein content (20.27%). Tannin content reduced significantly (37.72%) at the treatment of 4.5 mL of mixed microbes/100g rain tree seed meal with an incubation time of 96 hours. These findings suggest that rain tree seed meal, subjected to fermentation for 72 hours or more, improves nutritional quality, DMD, and OMD.

Keywords: Fermentation, in vitro, mixed microbes, nutrition, *Samanea saman*

Abbreviations: NFE: Nitrogen Free Extract, CFU: Colony Forming Unit, mL: Milliliter, DMD: Dry Matter Digestibility, OMD: Organic Matter Digestibility, DM: Dry Matter, OM: Organic Matter, ANOVA: Analysis of variance, SPSS: Statistical Package for the Social Sciences, RPM: Revolution per minute

INTRODUCTION

Feed in aquaculture is usually from feed aquaculture manufacture, generally consisting of soybean meal; soybean meal as a source of protein (Pham et al. 2010). According to Qin et al. (2022), soybean meal contains high protein with a balanced amino acid composition. Shrimp feed for *Litopenaeus vannamei* contains the highest composition of vegetable protein (30-35%) (Abu-Alya et al. 2021). It is one of the main determinants of the quality of aquaculture production (Nunes et al. 2022).

Currently, global food demands for soybeans are increasing as a source of food (Kim et al. 2021), feed (Vandenplas et al. 2021), and a raw material in the cosmetics industry (Majchrzak et al. 2022), which has an impact on increasing soybean prices. Therefore, aquaculture faces the challenge of finding alternative sources of vegetable protein for vaname shrimp feed (Murni et al. 2019; Zainuddin et al. 2019) that do not compete with human food needs. Moreover, it is important to explore various feed sources, one alternative being rain tree seeds. Rain tree seed meal contains a high protein content of 40.01%. However, rain tree seed has several disadvantages, including having a thick cell wall and a high crude fiber content of 11.72%, which inhibits feed digestibility and nutrient absorption (Hertrampf 2006). In addition, it contains anti-nutritional compounds such as 4% tannins. Shrimp feed is recommended to have tannin no more than 3% (Novriadi et al. 2021). High tannins in feed cause bitterness and astringency and form complex bonds with proteins that interfere with digestive enzyme activity, inhibiting organism growth (Hassan et al. 2020). Therefore, these limitations of rain tree seed utilization can be minimized through fermentation.

Fermentation converts organic matter into simpler compounds through enzymes produced by microorganisms (bacteria, protozoa, fungi/molds/fungi, yeast) (Dawood and Koshio 2020; Purwoko et al. 2023). These enzymes can simplify complex compounds into simpler ones, increase feed palatability, remove anti-nutritional substances, and increase nutritional value (Adelina et al. 2020). During the fermentation, *Bacillus* sp. produces amylase enzyme as much as 50.75 IU/g, lignocellulose 64.90 IU/g, cellulase 264 U/gds, pectinase 1.19 IU/g dan protease 1546.5 U mL^{-1} (Salim et al. 2017; Pham et al. 2022). At the same time, *Saccharomyces cerevisiae* produces enzymes protease, amylase, carboxypeptidase, aminopeptidase, and intervase (Fairbairn et al. 2021; Fernandes et al. 2021; Gronchi et al. 2022). *Rhizopus* sp. produces lipase, cellulase, hemicellulase, amylase, pectinase, and protease (Pathania et al. 2018; Fernandes et al. 2021; Jaworska et al. 2022).

Moreover, fermented soybeans in *Bacillus subtilis natto* have better nutritional value than unfermented soybeans by increasing the degree of protein hydrolysis by 177.1%, crude protein by 55.76%, and crude fiber decreased by 14.1% (Zhang et al. 2021). *Rhizopus* sp. and *Saccharomyces* sp. could degrade 89% of anti-nutrients, namely caffeine, into paraxanthine. At the same time, fermentation in *Saccharomyces cerevisiae* reduces tannin levels in sorghum by 87.6% (Purwoko et al. 2023). Previous research reported that seaweed fermented with mixed microorganisms *Bacillus* sp., *Rhizopus* sp., and *Saccharomyces* sp with composition of 1 mL + 1 g + 1 g 100 g^{-1} meal, increased protein content (9.23-15.93%), NFE (58.47-70.26%), DMD (24.86-27.76%), and OMD from (8.35-11.66%) (Aslamyah et al. 2017). However, research has never been conducted on fermented rain tree seed meal using mixed microbes to improve nutritional value and digestibility besides reducing anti-nutritions.

MATERIALS AND METHODS

Collection of rain tree seeds and preparation of rain tree meal

The seeds of rain tree used in this study were collected from Makassar, Indonesia. The ripened rain tree fruits that fall around the tree were peeled and sun-dried at 34-35°C for 3 hours to reach a dryness level of 90% and ground using a Philips Hr-2057 blender with a knife spinning speed of 30,000 rpm and then sifted through a 60-mesh sieve.

Microorganisms

The microbes used in this study consist of *Bacillus* sp., *Rhizopus* sp., and *Saccharomyces* sp. *Bacillus* sp. 0220000862736 was obtained from Micro Small and Medium Enterprises Biopocall Makassar-Indonesia, with a 4.82×10^6 CFU/mL density. *Rhizopus* sp. was obtained from Aneka Fermentasi Industri (AFI) Ltd Bandung-Indonesia with a density of 1.6×10^7 CFU/g. *Saccharomyces* sp. was purchased from Sangra Ratu Boga-Indonesia Ltd, with a density of 1.2×10^8 CFU/g.

Mixed microbes preparation

Each microbe was cultured separately: 2 mL of *Bacillus* sp. was cultured in the growth media containing 2 L of coconut water and 500 g of sugar and incubated in a glass jar at room temperature for 24 hours. *Rhizopus* sp. and *Saccharomyces sp*. were cultured by mixing 10 g of granulated sugar diluted with 100 mL of sterile aquadest and stirred using a magnetic stirrer at 120 rpm at room temperature for one hour.

The microorganisms were mixed using a method described by Aslamyah et al. (2017) with a modified composition, homogenized and diluted according to the treatment as follows: Treatment of mixed microbes in rain tree meal: (i) Treatment A (control) without the addition of microbes, (ii) Treatment B: mixed microbes consisting of 0.5 mL + 0.5 g + 0.5 g/100 g rain tree seed meal + 10.5 mL aquadest, (iii) Treatment C: mixed microbes consisting of 1 mL + 1 g + 1 g/100 g rain tree seed meal + 9 mL aquadest, (iv) Treatment D: mixed microbes consisting of 1.5 mL + 1.5 g + 1.5 g/100 g rain tree seed meal + 7.5 mL aquadest.

Fermentation process of rain tree seed meal

One hundred grams of rain tree seed meal were evenly mixed using a sprayer with the appropriate dose of mixed microbes, as specified by the respective treatment. Subsequently, the mixture was tightly sealed in plastic containers and subjected to anaerobic incubation. These plastic containers were stored in a closed box at a constant room temperature of 29°C throughout fermentation. After fermentation, the rain tree seed meal was subjected to heat treatment by immersing it in hot water (60°C) for one minute to stop the enzyme activity. The meal was allowed to cool to room temperature. Finally, the processed rain tree seed meal was analyzed at the Feed Chemistry Laboratory, Faculty of Animal Husbandry, Universitas Hasanuddin.

Research design

The experimental design in this study is a Factorial Completely Randomized Design, according to (Aslamyah et al. 2017). The first factor is the dose of mixed microbes, and the second factor is the length of incubation time.

The first factor is the treatment of a dose of mixed microbes in rain tree seed meal, namely: (A1) Without the addition of microbes (control), (A2) Addition of mixed microbes of 1.5 mL /100 g rain tree seed meal, (A3) Addition of mixed microbes of 3.0 mL /100 g rain tree seed meal, (A4) Addition of mixed microbes of 4.5 mL/ 100 g rain tree seed meal. Each treatment of a dose of mixed microbes has three replications.

The second factor is the length of incubation time: (B1) 48 hours incubation time, (B2) 72 hours incubation time, (B3) 96 hours incubation time. There are 12 treatments with 3 replications, so 36 experimental units were obtained.

Determination of the proximate composition of rain tree seed meal

Parameters observed for fermented grain meal include moisture, crude fiber, protein, fat, and NFE content, analyzed using the AOAC (Association of Analytical Chemists 2010) method (1).

Analysis of Dry Matter Digestibility (DMD) and Organic Matter Digestibility (OMD).

The dry and organic matter digestibility analysis refers to the method by (Minson and Leod 1972) and is calculated based on the formula (1) and (2) as follows:

DM weight of sample – (DM residue-blank)

$$DMD = \frac{\text{DM fresh sample (g)} - \text{DM residual sample (g)}}{\text{DM fresh sample}} \times 100\% \quad (2)$$

$$OMD = \frac{\text{OM fresh sample (g)} - \text{BO residual sample (g)}}{\text{OM fresh sample}} \times 100\% \quad (3)$$

Where: DM: Dry Matter, OM: Organic Matter, DM weight of the sample: sample weight x % DM, DM residue: Weight after oven-CP-filter paper, Blank: weight after oven-CP-filter paper, OM sample: DM weight of sample x % OM, % OM: 100% DM - (% Ash content in DM), OM residue: weight after oven – weight after tanur - the weight of filter paper

Dissolved protein levels

Dissolved protein was measured at the end of the observation following the method described by Lowry et al. (1951). 0.5 g of fermented rain tree seed flour was hydrolyzed to eliminate the reaction of crude protease and then mixed with 1.5 mL of 5% trichloroacetate and left at room temperature. Subsequently, 3 mL of Tris HCL at pH 6.5 was added, and the mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was used to analyze dissolved protein by using the 8400 Kjeltec Nitrogen Analyser (FOSS, Hoganas, Sweden) (4).

Tannin content

The tannin content of the sample was determined by the Folin Denis Colorimetric method, following (Nwosu et al. 2014). One g of processed rain tree meal was mixed with distilled water at 1:10 (W/V). The mixture was stirred for 30 minutes at room temperature and filtered to obtain the extract. A standard tannic acid solution was prepared: 2 mL of stock solution and was dispensed into 50 mL measuring flasks, while a blank sample using 2 mL of distilled. Then, 2 mL of each extract was put into separate flasks, each labeled accordingly. Then, each flask was added with 35 mL of distilled water, 1 mL of Folin Denis reagent, and 2.5 mL of saturated Na_2CO_3 solution. Afterward, each flask was diluted to a mark of 50 mL with distilled water and incubated for 90 minutes at room temperature. The absorbance was measured at 710 nm using a colorimeter (Jenway 6051) with the empty reagent at zero. The tannin content is calculated using the formula:

$$\% \text{ tannin} = 100/W \times a\_u/ a\_s \times C \times V\_t/ V\_ a \times D \quad (5)$$

Where: W: sample weight, a_u: absorbance of the samples, a_s: absorbance of standard tannin solution, C: Concentration of standard tannin solution, V_t: Total volume of extract, V_a: volume of extract that was analyzed, D: dilution factor

Data analysis

Proximate (crude fiber, proteins, fat, NFE), DMD, OMD, dissolved proteins, and tannin levels; each treatment was analyzed using a variety of fingerprints. If there is a difference between treatments, proceed with the Duncan test at a 95% confidence interval using the SPSS program version 20. Next, the graph was constructed using Origin (Origin 2023b USA).

RESULTS AND DISCUSSION

Results

The effect of mixed microbes dose and incubation time on the proximate composition of rain tree seed meal

The proximate composition of rain tree seed meal fermented using mixed microbes is presented in Table 1. The dosage of mixed microbes and length of incubation time using ANOVA significantly affect the proximate analysis of fermented raint ree seed meal (P<0.05), and there is a reaction between the dosage of the mixed microbes and the length of incubation. Administration of the microbial mixture at a dose of 4.5 mL/100 g, with an incubation time of 72 hours, produced the highest levels of crude protein (44.94%) and NFE (44.44%), with the lowest values of crude fiber (4.12%) and fat (0.97%). These results showed statistically significant differences from other treatment results (P<0.05). Furthermore, the control treatment produced the lowest percentage of crude protein (40.26%) and NFE (41.54%) and had the highest concentration of crude fiber (10.20%) and fat (3.62%). The values observed in this treatment were statistically different from those observed in the other treatments (Table 1).

The effect of mixed microbes dose and incubation time on dry matter digestibility and organic matter digestibility in vitro

The results showed that the mixed microbes dose and incubation time significantly affected fermented rain tree seed meal's dry matter and organic matter digestibility (P<0.05), and there is an interaction between the dose of mixed microbes and incubation time. The highest dry matter digestibility (75.74%) and organic matter (69.54%) of rain tree seed meal was obtained at the treatment of mixed microbes dose of 4.5 mL/100 g with an incubation time of 72 hours, significantly higher than other treatments. Dry matter digestibility (55.36%) and organic matter digestibility (46.23%) of the control treatment were significantly lower than other treatments (Figures 1.A and 1.B).

The effect of mixed microbes dose and incubation time on dissolved protein

The degradation process of rain tree seed meal by mixed microbes could be measured from dissolved protein contents. The dissolved protein content of rain tree seed meal increases with increasing doses of mixed microbes but not with the increased incubation time. The highest dissolved protein content of rain tree seed meal (89.76%) was obtained at a treatment of mixed microbes dose of 4.5 mL/100 g and incubation time of 72 hours, significantly higher than other treatments (P<0.05). The lowest content of dissolved protein (74.75%) was obtained in the control treatment, with an incubation time of 48 hours significantly lower than other treatments. An interaction between dose and incubation time affected the dissolved protein in rain tree seed meal (Figure 2).

The effect of mixed microbes dose and incubation time on the content of tannin

The results showed that doses of mixed microbes, incubation time, and the interaction of both significantly affected ($p<0.05$) the reduction of tannin content of rain tree seed meal. The highest tannin was obtained in the control treatment with 48 h of incubation (Figure 3).

Table 1. Crude fiber, protein, fat, and NFE content of fermented rain tree seed meal

Treatments	Moisture (%)	Crude fiber (%)	Protein (%)	Fat (%)	NFE(%)
A1_B1	7.70±1.07^{c}	10.20±0.015^{l}	40.26±0.00^{a}	3.62±0.00^{k}	41.54±0.01^{a}
A1_B2	5.53±0.01^{a}	9.23±0.20^{k}	40.36±0.00^{b}	3.60±0.00^{j}	42.46±0.02^{b}
A1_B3	6.91±0.01^{b}	9.19±0.10^{k}	40.38±0.05^{c}	3.58±0.00i	42.51±0.01^{c}
A2_B1	6.87±0.01^{b}	8.55±0.03^{j}	41.07±0.01^{d}	3.02±0.00^{h}	43.03±0.03^{d}
A2_B2	7.21±0.01^{b}	8.22±0.01^{i}	41.88±0.00^{e}	2.97±0.01^{g}	42.64±0.01^{e}
A2_B3	8.13±0.05^{c}	8.12±0.01^{h}	42.04±0.00^{f}	2.67±0.00^{f}	42.68±0.01^{f}
A3_B1	6.97±0.02^{b}	7.30±0.09^{f}	42.95±0.00^{g}	2.34±0.00^{e}	43.16±0.09^{g}
A3_B2	6.92±0.03^{b}	6.56±0.03^{e}	43.01±0.00^{h}	2.20±0.00^{d}	44.00±0.03^{h}
A3_B3	6.69±0.01^{b}	6.32±0.02^{d}	43.92±0.00^{i}	2.11±0.00^{c}	43.43±0.02^{i}
A4_B1	6.64±0.03^{b}	6.11±0.01^{c}	44.56±0.00^{j}	1.13±0.00^{b}	44.02±0.01^{i}
A4_B2	5.95±0.03^{a}	4.12±0.00^{a}	44.94±0.00^{l}	0.97±0.00^{a}	44.44±0.01^{k}
A4_B3	6.68±0.01^{b}	5.10±0.00^{b}	44.80±0.00^{k}	1.12±0.00^{b}	44.37±0.01^{j}

Note: A1_B1: Control_48 hours; A1_B2: Control_72 hours; A1_B3: Control_96 hours; A2_B1: Dose 1.5 mL/L_48 hour; A2_B2: Dose 1.5 mL/L_72 hours; A2_B3: Dose 1.5 mL/L_96 hours; A3_B1: Dose 3 mL/L_48 hours; A3_B2: Dose 3 mL/L_72 hours; A3_B3: Dose 3 mL/L_96 hours; A4_B1: Dose 4.5 mL/L_48 hours; A4_B2: Dose 4.5 mL/L_72 hours; A4_B3: Dose 4.5 mL/L_96 hours

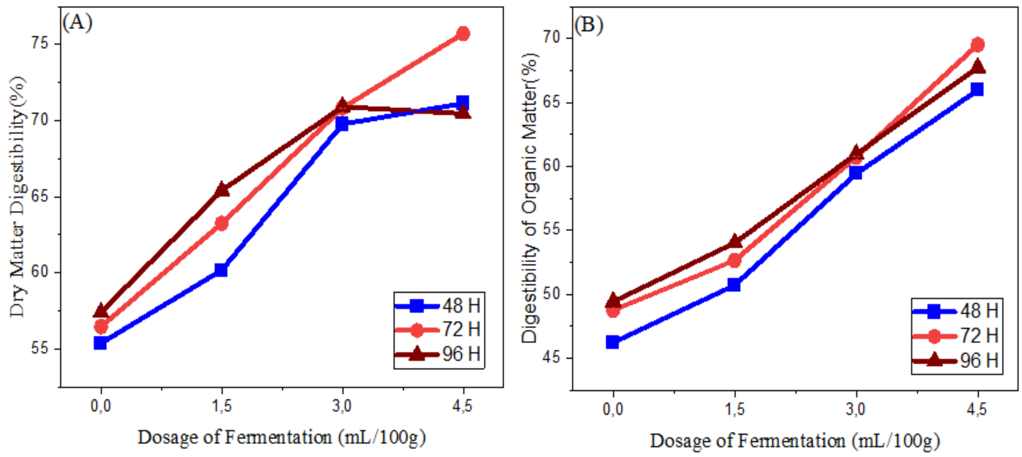

Figure 1. (A) Dry matter digestibility and (B) organic matter digestibility of fermented rain tree seed meal

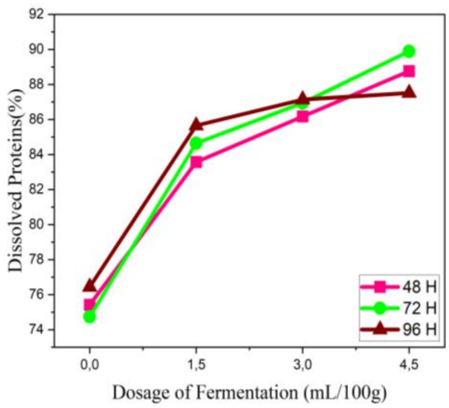

Figure 2. The percentage of dissolved protein in fermented rain tree seed meal

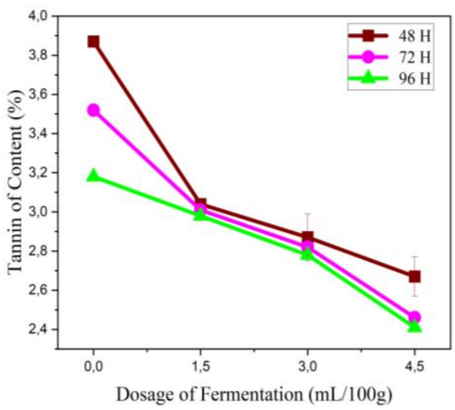

Figure 3. Tannin content of fermented rain tree seed meal

Discussions

Proximate composition of fermented rain tree seed meal

The treatment of mixed microbes with a dose of 4.5 mL/100 g and an incubation time of 72 hours resulted in the lowest crude fiber content (4.12%), similar to the reduction of 59.60% (Table 2). It might be attributed to the higher dose of mixed microbes than other treatments and the higher population of microbes, which might increase cellulase enzyme activity. A study by Mirnawati et al. (2019) suggests that higher doses of microorganisms accelerate the fermentation process due to increased microbial growth and enzyme activity. However, at 96 hours of incubation, the 4.5 mL/100 g dose decreased crude fiber content. The decrease of the treatment of 96 h incubation was lower than that of 72 h incubation time. It might reduce viable microorganism cells, possibly due to a depletion of nutrient sources required for cell growth. The increasing incubation time increases degraded substrate by enzymes produced by mixed microbes. The growth of microorganisms declined after reaching an optimal point. Previous studies (Yunilas et al. 2019; Wang et al. 2022; Baena et al. 2022) emphasized that interactions among substrates, incubation periods, and the types and quantities of enzymes influence the degradation of crude fibers. The fermentation rate significantly affects enzymes produced in the medium (Pongsetkul et al. 2022).

The absence of a microbe mixture prevented the degradation process, resulting in a higher crude fiber content (10.20%) at the control treatment and an incubation time of 48 hours. The low degradation of crude fiber was obtained at the treatments of 1.5 and 3 mL mixed microbes/100 g with various incubation periods of 48, 72, and 96 hours, possibly due to a lower mixed microbe population, resulting in lower cellulase enzyme activity. This study demonstrates that a combination of 4.5 mL/100 g of mixed microbial dose and an incubation period of 72 hours was the most effective combination for promoting the development of mixed microorganisms, specifically *Bacillus* sp., *Rhizopus* sp., and *Saccharomyces* sp., which are capable of creating cellulase enzymes. However, the growth starts to decline at the incubation time of 96 hours. It demonstrates that the level of enzyme activity is initially low but gradually rises as the fermentation process advances. Eventually, as microbial growth diminishes, the enzyme activity also declines. Enzyme activity often follows a sequence of growth patterns, i.e., adaptation, rapid growth, stability, and a decrease in growth (Graf and Buchhaupt 2022). Enzyme activity typically follows a growth pattern, transitioning through adaptation, exponential growth, a stationary phase, and followed by a decline in growth.

Crude fiber represents the indigestible portion of carbohydrates and is not considered an essential nutrient for shrimp. The dietary fiber requirement in shrimp feed does not exceed 10% (Jannathulla et al. 2017). The results of this study showed a 40.39% reduction in crude fiber content (from 10.20 to 4.12%) at the treatment of mixed microbes of 4.5 mL/100 g and an incubation time of 72 hours, suggesting that rain tree seed meal could be suitable for shrimp feed. Previous research showed that fermentation of sago pulp using cellulase enzyme (0.75 g/kg) reduced crude fiber content from 12.79 to 7.91% (Zulkarnain et al. 2016). Similarly, the crude fiber in *kepok* banana stems decreased from 17.82 to 15.46% when fermented with 5% cellulase and incubated for 7 days (Hidayat et al. 2022). The conversion of fiber components to simpler compounds is primarily due to the action of endoglucanase in cellulase enzymes, breaking glycosidic β-1,4 bonds in cellulose into simpler compounds such as glucose that are quickly digested.

The highest protein content in rain tree seed meal was obtained at a treatment of mixed microbes dose of 4.5 mL/100 g with an incubation period of 72 hours, but slightly decreasing at 96 hours of incubation (44.80%) in protein content. It might be caused by higher protease production from *Bacillus* sp and *Rhizopus* sp. Prior studies have demonstrated that protease enzyme levels rise proportionately to the number of peptide bonds cleaved in proteins (Razavizadeh et al. 2022). This process produces simple peptides with high protein solubility (Yarlina et al. 2020; Cruz-Casas et al. 2021). Additionally, increased proteolytic enzyme concentration during hydrolysis leads to higher dissolved nitrogen content in protein hydrolysates (Aslamyah et al. 2022).

Lower protein content in rain tree seed meal was observed in treatments with mixed microbe doses of 1.5 and 3 mL/100 g and incubation times of 48 and 72 hours, compared to the 4.5 mL/100 g dose with a 72 hour incubation time. It can be attributed to the lower dose of mixed microbes, which required more time to hydrolyze the protein in rain tree seed meal. The 96 hour incubation time might be an excessive incubation period that may deplete the substrate necessary for protease enzyme activity. Researchers have reported that the ratio between enzymes and substrates directly affects the breakdown of complex compounds into simpler ones (Zhu et al. 2021; Zhang et al. 2022).

Protein degradation by enzymes involves the breakdown of covalent bonds connecting amino acid molecules in proteins, forming free amino acids (Dinakarkumar et al. 2022). Various factors influence protein hydrolysis, including substrate-to-enzyme ratio, pH, enzyme type, temperature, and hydrolysis time. Dissolved nitrogen increases as the protease enzyme concentrations increase during hydrolysis (Fang et al. 2022). Moreover, hydrolysis breaks down insoluble complex protein compounds into simpler compounds, such as amino acids, ammonia, and peptides, which are more easily solubilized (Yang et al. 2020).

The highest crude fat degradation was observed in the mixed microbe dose of 4.5 mL/100 g treatment with a 72 hour incubation, resulting in a fat content reduction of 73.20% (from 3.62 to 0.97%) (Table 2). It indicated optimal hydrolysis conditions (Alahmad et al. 2022); the hydrolyzed fat will increase with increasing hydrolysis time until it reaches a stationary state and shows a linear value. The 72 hour incubation indicated optimal hydrolysis. There is an increase in the degree of hydrolysis. In the 96 hour incubation, the percentage of fat reduction was lower than that of the 72 hour incubation.

Fat is an energy source and plays a crucial role in maintaining the integrity of biological membrane structures. Fat in feeds is a source of essential fatty acids, phospholipids, sterols, and carotenoids necessary for growth, survival, and metabolism. The fat content significantly affects the shelf life and stability of hydrolysate products, particularly regarding resistance to fat oxidation. Protein hydrolysate products with lower fat content are generally more stable and durable than those with higher fat content (Shi et al. 2015).

The main component of rain tree seed flour is the Nitrogen Free Extract (NFE), which consists mainly of glucose derived from the breakdown of cellulose during hydrolysis. Glucose, the most basic form of carbohydrate, can be quickly metabolized by living organisms. Hydrolyzed rain tree seed meal has a good nutritional composition and is a promising source of shrimp feed. Treatment with a dose of 4.5 mL mixed microbes and a 72-hour incubation resulted in the highest degree of NFE, reaching 44.44%, with a percentage reduction of 6.52% (from 44.44 to 41.54%) (Table 2). It is likely due to the increased activity of cellulase enzymes with increasing doses of mixed microbes and longer fermentation time, thereby accelerating the growth and development of mixed microbes. Previous research reports that higher inoculum doses create a more conducive environment for microbial growth, accelerating fermentation processes and enhancing enzyme activity (Senanayake et al. 2023). The duration of fermentation plays a critical role because longer fermentation allows increased substrate hydrolysis by microorganism-produced enzymes (Mirnawati et al. 2019).

The role of mixed microbes to improve dry matter digestibility and organic matter digestibility in vitro

The digestibility of dry matter and organic matter serves as an indicator of feed quality. Higher digestibility in feed corresponds to better quality feed (Hernaman et al. 2022; Selim et al. 2022). Dry matter digestibility, in vitro, indicates dry matter that microorganisms can digest. In contrast, organic matter digestibility indicates the amount of organic matter digested by enzymes produced by microorganisms (Hernaman et al. 2015). Digestibility means the nutritional value of feed ingredients available for organisms (Hendarto et al. 2022). The composition of feed ingredients and treatment affects the digestibility of dry and organic matter. A high level of digestibility in feed suggests that certain nutrients make a substantial impact. Low digestibility indicates that these compounds do not supply essential nutrients for development and productivity (Sousa et al. 2023). The quality of feed is determined by feed digestibility, with higher dry matter and organic matter digestibility indicating better utilization (Wang et al. 2021). Feed quality is categorized based on digestibility levels, i.e., 50-60% considered low quality, 60-70% medium quality, and above 70% high quality (Kearl 1982).

The results in this study indicate that the treatment increased dry matter digestibility from 55.37 to 75.74%, while organic matter digestibility increased from 46.23% to 69.54% (Figure 1). The highest digestibility was obtained from the treatment of a mixed microbe dose of 4.5 mL and an incubation time of 72 hours. The increase in digestibility can be attributed to the higher amount of mixed microbes than other treatments, suggesting a synergistic effect from the extracellular enzymes produced by the mixed microbes, including protease, amylase, lipase, pectinase, lignocellulose, carboxypeptidase, aminopeptidase, and invertase. This higher enzyme activity resulted in enhanced digestibility. In contrast, lower doses of mixed microbes (1.5 and 3 mL) resulted in lower digestibility of organic and dry matter due to a decrease in enzyme activity, requiring a more extended incubation period and suboptimal fermentation (Liñan-Vidriales et al. 2021; Zhang et al. 2021; Harlina et al. 2021). The control treatment had lower digestibility due to the absence of the degradation process of complex compounds and the absence of enzyme-producing microorganisms. Moreover, an incubation time of 96 hours at a mixed microbe dose of 4.5 mL showed decreased hydrolysis, which might be due to prolonged incubation, resulting in reduced substrate availability and an imbalanced microorganism population. Previous studies (Murtius et al. 2022; Zhu et al. 2022) showed that enzyme activity dynamics are influenced by dose and incubation time.

The digestibility of dry and organic matter in this study is higher than those reported in previous studies. A study by Lunagariya et al. (2017) on mixed diets enhanced with external additives showed that the fibrinolytic enzymes resulted in a dry matter digestibility of 63.03% and an organic matter digestibility of 63.62%. Aslamyah et al. (2017) showed that seaweed fermentation using a combination of microorganisms produces dry matter and organic matter digestibility of 60.92 and 57.77%, respectively. Murni et al. (2018) fermented vegetable waste using rumen fluid has a dry matter content of 27.76% and an organic matter content of 11.66%. A previous study by Fattah et al. (2020) on green concentrate fermentation produces dry matter and organic matter digestibility of 42 and 38.69%. Maranatha et al. (2020) fermented corn cobs and moringa leaf flour using EM_4, resulting in dry matter and organic matter digestibility of 67.94 and 66.77%, respectively. Vargas-Ortiz et al. (2022) reported that using *Acacia mearnsii* (AM) in feed yielded a dry matter digestibility of 69.5% and an organic matter digestibility of 66.9%. Those different results are due to different types of substrates and enzymes.

The role of mixed microbes in increasing dissolved proteins

Due to their simplified molecular structure, the digestive system quickly absorbs dissolved proteins composed of oligopeptides or amino acids (Salem et al. 2022). Dissolved protein content in fermented rain tree seed meal was increased from 74.73 to 89.88% at a mixed microbe dose of 4.5 mL/100 g with an incubation time of 72 hours due to the combined action of mixed microbes, i.e., *Bacillus* sp, *Saccharomyces* sp. and *Rhizopus* sp. which secrete protease enzymes (Zhang et al. 2021; Hawar 2022). Protease enzymes produced by these microbes convert the proteins of rain tree seed meal into peptides or free amino acids and increase solubility. Increasing soluble protein resulted from breaking down complex molecules into simpler ones and water-soluble micromolecules during fermentation. However, the extended incubation time to 96 hours at the same dose of mixed microbes caused a slight decrease in dissolved protein levels from 89.88 to 87.51%. This reduction may be due to excessive incubation times, leading to disrupted microorganism growth due to nutrient depletion and gradual cell death during fermentation.

Increased dissolved protein levels correlate with reduced tannin levels during fermentation. A negative correlation exists between tannin reduction and increased dissolved protein. The decrease in tannin levels resulted in an equivalent rise of 118.42% in dissolved protein compared to the initial. The remaining variability was attributed to other factors (Figure 4).

Soluble protein testing assesses the over- or under-processing of rain tree seed meal and evaluates protein denaturation, which can reduce protein utilization. The dissolved protein in rain tree seed meal before fermentation was 75.40% and increased by 13.4% after fermentation, indicating that rain tree seed meal was not excessively fermented and protein breakdown was effective. Protease enzymes hydrolyze peptide bonds in polypeptide chains via endopeptidase (Christensen et al. 2022). Previous studies showed that commercial feed fermentation using mixed microbes, i.e., *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, and *Bacillus safensis* at a density of 10^8 CFU/mL, was more effective in increasing dissolved protein and essential amino acid content (lysine and methionine) than single-strain fermentation (Zhang et al. 2021). Fermentation of soybean using *Bacillus subtilis natto* increased dissolved protein by 17.5% (Zhang et al. 2021).

Table 2. Nutritional value of fermented rain tree seed meal at different doses of mixed microbes

Parameter	Percentage (%)	Treatment	Percentage (%)	Treatment	Percentage of change (%)
Crude fiber	4.12	A4B2	10.20	A1B1	59.60
Proteins	40.26	A1B1	44.94	A4B2	11.62
Fat	0.97	A4B2	3.62	A1B1	73.20
NFE	41.54	A1B1	44.44	A4B2	6.52
Dry matter digestibility	55.37	A1B1	75.74	A4B2	36.78
Organic matter digestibility	46.23	A1B1	69.54	A4B2	50.42
Dissolved proteins	74.73	A1B1	89.88	A4B2	20.27
Tannin	2.41	A1B1	3.87	A4B3	37.72

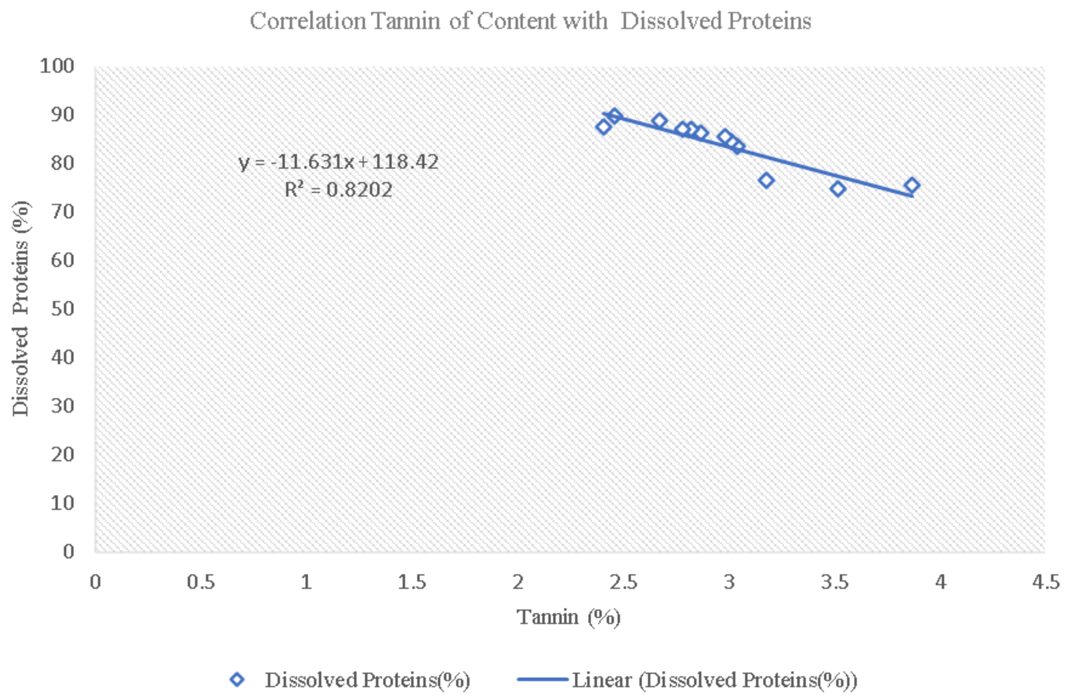

Figure 4. Correlations between tannin content with dissolved proteins in fermented rain tree seed meal using mixed microbes

The role of mixed microbes in tannin degradation

Tannins are polyphenolic compounds that bind proteins, inhibit protease enzymes, and form complex proteins that cannot be digested. They limit protein availability and reduce digestibility (Olawoye and Gbadamosi 2017); therefore, tannins content in fish feed should not exceed 3% (Vargas-Ortiz et al. 2022).

The results showed that the lowest reduction in tannin content (2.41%) was obtained at a treatment dose of 4.5 mL mixed microbes/100 g with an incubation period of 96 hours. The tannin level in the control treatment was 3.87%, so this research reduced 37.72% (Table 2). compared to the initial tannin content. This was caused by increasing the dose and the incubation time for the mixture of microbes, including *Rhizopus* sp. and *Saccharomyces* sp., which is a more effective producer of the tannase enzyme than other treatments. Lactic acid bacteria, namely *Rhizopus* sp. and *Saccharomyces* sp., hydrolyze sugars and carbohydrates in rain tree seed flour, causing tannin hydrolysis due to the carbohydrate groups in the tannin molecular structure. Previous research reported that spontaneous fermentation of proso millet (*Panicum miliaceum*) flour reduced tannin content by 1.51% (Mahendra et al. 2019). In addition, *Rhizopus* sp. is a good producer of the tannase enzyme and reduces tannin in jack beans (*Canavalia ensiformis*) at a dose of 0.02 g/100 g and an incubation period of 26 hours (Ramli et al. 2021). The tannin content of mugua fruit was reduced by 78% through fermentation using *Lactobacillus* sp. with a dose of 1 g with an incubation time of 34 hours (Shang et al. 2019).

This study concludes that (i) dose and incubation time and interaction of dose and incubation time influence both have a significant effect ($P<0.05$) on proximate composition, dry matter digestibility, organic matter digestibility, dissolved protein, and tannin content of rain tree seed meal. Treatment of mixed microbes dose of 4.5mL/100 g and incubation time of 72 hours reduced the highest crude fiber content by 59.60%, crude fat by 73.20% and increased crude protein content by 11.62%, Nitrogen Free Extract by 6.52%, dry matter digestibility by 36.78%, organic matter digestibility 50.42%, and dissolved protein 20.27%. Tannins content was reduced by 37.72% at mixed microbes dose of 4.5 mL/100 g incubation at 96 hours. (ii) The concentration of mixed microbes of 4.5 mL/100 g rain tree seed meal with an incubation time of 72 hours was the best treatment in improving the quality of rain tree seed meal.

ACKNOWLEDGEMENTS

We thank Universitas Hasanuddin, Makassar, Indonesia and Universitas Muhammadiyah Makassar, Indonesia for allowing and facilitating the conduct of this research.

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