Research Article

Improving *Azolla microphylla* through Fermentation with Lignocellulolytic Fungi and its Application in Broiler Feed

Nuraini Nuraini*, Mirzah Mirzah, Yuliaty Shafan Nur, Harnentis Harnentis

Department of Feed Animal and Nutrition, Faculty of Animal Science, Universitas Andalas, Padang, Indonesia.

Abstract | *Azolla microphylla* has advantages as feed ingredients for poultry because it proliferates and has high protein but contains high fiber. Therefore, fermentation with lignocellulolytic fungi was carried out to improve the nutritional quality of *Azolla microphylla*. This research has 2 phases. Phase 1, determination of the best types of lignocellulolytic fungi on the nutrient quality of fermented *Azolla microphylla*. This study used an experimental method with a Completely Randomized Design (CRD). The treatments were the types of fungi, namely *Lentinus edodes, Pleurotus ostreatus,* and *Phanerochaete chrysosporium,* and six replications. The parameter measured: cellulase activity, crude fiber, fiber digestibility, crude protein, and nitrogen retention. The results of phase 1 showed that the types of fungi had a very significant effect (P <0.01) on the nutrient quality of fermented *Azolla microphylla*. Fermentation of *Azolla microphylla* with *Lentinus edodes* and *Pleurotus ostreatus* gave the best products. Phase 2. Application Fermented *Azolla microphylla* (AMF) in the diet on broiler performance. Fermentation with *Lentinus edodes* has high amino acids, especially glutamic acid, increasing the palatability of broiler . The research method used a completely randomized design (CRD) consisting of 5 treatments and four replications. The treatments were the use of AMF in the diet: 0%, 10, 15, 20, and 25% AMF. The results of phase 2 showed that the treatment had a very significant effect (P<0,01) on feed consumption, body weight gain, carcass percentage, and carcass meat taste but gave no considerable impact (P>0,05) on feed conversion and abdominal fat percentage. The conclusion of phase 1 was the fermentation of *Azolla microphylla* with *Lentinus edodes,* and *Pleurotus ostreatus* gave the best products. The decision of phase 2, the use of *Azolla microphylla* fermented with *Lentinus edodes,* can be used up to 20% in the diet and can maintain the performance of broilers.

Keywords | *A. microphylla*, Fermentation, Lignocellulolytic fungi, Performance, Broiler

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***Correspondence** | Nuraini Nuraini, Department of Feed Animal and Nutrition, Faculty of Animal Science, Universitas Andalas, Padang, Indonesia; **Email:** nuraini@ansci.unand.ac.id
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INTRODUCTION

Broilers have a relatively short harvest period and can guarantee the availability of meat and meet the needs of the Indonesian people for nutrition. One of the determining factors for the success of a broiler farming business is the feed factor, genetic factors, and maintenance management. The cost of feed in the livestock business, especially broilers, is the most significant component of the total production costs that farmers must incur during the production process, around 60-70% (Amrullah, 2004). Chickens can grow and produce optimally with the maximum profit level for the broiler farming business to succeed. The feed factor must receive serious attention.

Protein source feed ingredients used by farmers are generally still imported, such as soybean and fish meals. One effort to reduce feed costs is to use alternative feed ingredients. Measures can be made by utilizing local feed ingredients that already exist as protein sources, such as

water spikes (*Azolla microphylla*). This fern plant is an aquatic plant with small green leaves and can be cultivated in ponds of the required size.

Azolla microphylla has the advantage of being a feed ingredient for poultry, namely its high protein content of 20-35%. It contains vitamins A and B12 and amino acids (lysine 0.46%, methionine 0.05%, and amino acid glutamate 1.54% (Nuraini and Mirzah, 2021). Growth *Azolla microphylla* is relatively fast, i.e., it takes 2-9 days to multiply, and 20 tons of fresh biomass/ha can be obtained from seeds of 0,5 tons/ha. Production biomass *Azolla microphylla* is relatively high, i.e., the weight reaches 1-2 kg/m2 depending on the fertility ponds (Lukiwati et al., 2008).

The nutritional content of flour *Azolla microphylla,* according to Lukiwati et al. (2008), reported that crude protein 23.7%, crude fiber 15%, crude fat 2.93%, Ca 2.07%, P 0.77%, metabolic energy of 2160 kcal/kg, and various amino acids. Only up to 5% in the diet, Azolla flour can be used for better production in broilers due to the high crude fiber content in *Azolla microphylla* 29.83% (cellulose 17.36%, and lignin 22.64%) (Nuraini and Mirzah, 2021).

One of the efforts to improve the nutritional quality of *Azolla microphylla* requires fermentation technology. Fermentation is a desirable biochemical modification of microorganisms and their enzymes (Kahajdova and Karovicova, 2007). Fermentation can be carried out using fungi such as *Lentinus edodes, Pleurotus ostreatus* and *Phanerochaete chrysosporium.*

Lentinus edodes can degrade lignin and cellulose. It produces lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Mata et al., 2016; Nagai, 2002; Nuraini et al., 2017), cellulase (Elisashvili et al., 2008; Nuraini et al., 2017), protease (Azefedo et al., 2014; Souza et al., 2016). According to Nuraini et al. (2017), fermentation of palm oil sludge with *Lentinus edodes* at an inoculum dose of 8% and fermentation time of 11 days is the best treatment for decreasing crude fiber.

Pleurotus ostreatus is one of the lignocellulolytic microbes because it can degrade crude fiber cellulose and lignin components. *Pleurotus ostreatus* produces extracellular ligninolytic enzymes such as lignin peroxidase, Mn peroxidase, and laccase (Fernandez-Fueyo et al, 2016; Nuraini et al., 2017; Trisna et al., 2020; Nuraini et al., 2020). It can produce cellulase enzymes (Nuraini et al., 2017, 2020; Trisna et al., 2020) protease enzymes (Machado, 2016). The interaction of an inoculum dose of 8% and a fermentation time of 9 days is the optimal condition of a mixture of the cocoa pod and rice bran fermented with *Pleurotus ostreatus* (Nuraini et al., 2019b).

Phanerochaete chrysosporium produces ligninase and cellulase enzymes. *Phanerochaete chrysosporium* is a white-rot fungus known for its ability to degrade lignin (Howard et al., 2003). Fermentation using *Phanerochaete chrysosporium* mold with 70% durian skin and 30% tofu waste increases the crude protein by 65.13% with an inoculum dose of 8% and fermentation time of 9 days (Nuraini et al., 2015).

Increased crude protein (amino acids) and decreased crude fiber from *Azolla microphylla* fermented are expected to increase its use in the diet and positively affect the performance of broilers. What is the level limit, and how does it affect Azolla microphylla fermented use in the diet on broiler performance is unknown?

MATERIALS AND METHODS

Phase 1 Determination of the best types of lignocellulolytic fungi on the nutrient quality of fermented *Azolla microphylla*

Azolla microphylla fermentation

Substrate consists of 80% Azolla and 20% rice bran mixture, then homogenized. After that, sterilization was carried out with an autoclave (temperature 121°C for 15 minutes), then left until the temperature dropped to room temperature (25-30°C). The substrate inoculated with 8% fungi, namely A= *Lentinus edodes*, B= *Pleurotus ostreatus*, and C= *Phanerochaete chrysosporium*. Then added with Brook's solution 7ml/100g of the substrate. Then incubated for nine days in an incase. After harvesting the fermented product, its fresh weight weighed 10 grams per sample taken to analyze cellulase activity. After that, the fermented product was put in an oven (temperature 80°C for 2 hours) to turn off the fungus, then continued drying at 60°C for 8 hours.

Experimental design

This study used an experimental method with an experimental design that was a completely randomized design (CRD) with three treatments and six repetitions. The treatments were fungi, namely A= *Lentinus edodes*, B= *Pleurotus ostreatus*, and C= *Phanerochaete chrysosporium*.

Variable

The variable measured were: cellulase activity (U/ml), crude fiber (%), crude protein (%), nitrogen retention (%) and fiber digestibility (%).

Data analysis

All data is processed statistically by analyzing diversity according to the Completely Randomized Design (CRD) pattern. The differences between the treatments tested by the Duncan Multiple Range Test (DMRT) according to Steel and Torrie (1995).

Phase 2 Application *Azolla microphylla* fermented (AMF in the diet on broiler performance

Azolla microphylla fermentation

The substrate consists of 80% Azolla and 20% rice bran, then homogenized. After that, sterilization was carried out with an autoclave (temperature 121°C for 15 minutes), then left until the temperature dropped to room temperature (25-30°C). We were inoculated with 8% *Lentinus edodes*, then added 7ml/100g of the substrate with Brook solution. It was incubated for nine days. After harvesting the fermented product, its fresh weight weighed 10 grams per sample taken to analyze cellulase enzyme activity. After that, the fermented product was put in an oven (temperature 80°C for 2 hours) to turn off the fungus, then continued drying at 60°C for 8 hours.

Data analysis

All data is processed statistically by analyzing diversity according to the Completely Randomized Design (CRD) pattern. The differences between the treatments tested by the Duncan Multiple Range Test (DMRT) according to Steel and Torrie (1995).

The feeds used in this study were prepared using feed ingredients such as cornmeal, meat flour, soybean meal, Bravo 311, *Azolla microphylla* fermented (AMF), rice bran, coconut oil, and top mix. The feed ingredients, nutrient content, and metabolic energy (as feed) are listed in Table 1.

Table 1: Feed ingredients, nutrient content (%), and metabolic energy (kcal/kg) that make up the feed (as feed).

Feed ingredients	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ca (%)	P (%)	M.E. (Kcal/kg)[b]
Corn meal	8.20	2.66	3.50	0.38	0.19	3300
Meat flour	52.15	6.80	3.00	3.67	1.28	2150
Soybean meal	43.43	2.49	3.50	0.63	0.36	2240
Bravo 511[c]	22.50	5.00	5.00	0.90	0.60	3100
AMF[d]	27.26	7.20	15.81	0.21	0.11	2506
Rice bran	9.50	5.09	13.50	0.69	0.26	1630
Coconut oil [b]	-	100.00	-	-	-	8600
Top mix[e]	-	-	-	0.06	-	-

Information: a: Nuraini et al. (2020); b: Scott et al. (1982); c: Product Packaging Labels of PT. Charoen Pokphand; d: Nuraini et al. (2021); e: Product Packaging Labels of PT. Medion; AMF= *Azolla microphylla* Fermented.

Research methods

Experimental design

This study used an experimental method with a completely randomized design (CRD) with five treatments and four replications. The treatment are feeds containing AMF, namely: 0%, 10%, 15%, 20% and 25% AMF. We prepared the diet iso-protein 21.3% and iso-energy 2900 kcal/kg as Scott et al. (1982) recommended. The composition of the research diet is shown in Table 2. The nutritional content and energy of the research are listed in Table 3.

Measured parameter

Parameters measured were:

1. Feed consumption (g/head)
2. Weight gain (g/head)
3. Feed conversion
4. Carcass percentage
5. Abdomen fat percentage
6. Carcass meat taste test

Table 2: Research feed composition.

Ingredients	Treatment				
Feed	A	B	C	D	E
Ground corn	48.50	45.25	43.55	42.00	40.50
Meat flour	14.00	14.00	14.00	14.00	14.00
Soybean meal	15.60	10.25	7.60	4.80	2.25
Bravo 511	11.00	11.00	11.00	11.00	11.00
AMF	0.00	10.00	15.00	20.00	25.00
Fine bran	8.40	7.00	6.35	5.70	4.75
Coconut oil	2.00	2.00	2.00	2.00	2.00
Top mix	0.50	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00	100.00

Description: AMF (*Azolla microphylla* Fermented).

Fermentation of *Azolla microphylla* with *Lentinus edodes*

The substrate composition consists of 80% Azolla: 20% rice bran. Then it was sterilized by autoclave (121°C temperature for 15 minutes), after that allowed to cool down to room temperature (25-30°C). We inoculated with 8% inoculum *Lentinus edodes* of the substrate. And then added Brook's solution 7 ml/100g substrate. It stirred until evenly distributed, put into a basket container, covered with plastic, and then incubated for nine days in the incase. The fermented products are ready to be harvested, weighed fresh, and then dried in the sun and ground.

Data analysis

All data obtained were statistically processed by diversity analysis. Difference between the treatments tested with Multiple Range Test (DMRT). According to the procedure Steel and Torrie (1995).

RESULTS AND DISCUSSION

Phase 1 effect of treatment on cellulase activity, fiber decrease, and fiber digestibility

The effect of the fungi on the nutrient quality of *A.*

microphylla is the list in Table 4. The results of the statistical analysis showed that the type of fungi affected highly significantly (P<0.01) cellulase activity, fiber decrease, and fiber digestibility of *A. microphylla* fermented. The result of the DMRT test showed that the cellulase activity crude fiber decrease and crude fiber digestibility of fungi *Pleurotus ostreatus* (treatment A) and *Lentinus edodes* (treatment B) are significantly higher (p<0.01) than *Phanerochaete chrysosporium* (treatment C). but the crude fiber decrease in treatment A and B is significantly decrease (p<0.01) than treatment C.

Table 3: Nutrient content and energy metabolism of research feeds.

Nutrient content (%) and metabolic energy (kcal/kg)	Treatment				
	A	B	C	D	E
Crude protein	21.33	21.33	21.34	21.30	21.34
Crude fat	5.61	6.04	6.25	6.47	6.68
Crude fiber	4.35	5.44	5.99	6.54	7.06
Ca	0.95	0.92	0.90	0.88	0.87
P available	0.42	0.40	0.39	0.38	0.37
Amino acid glutamate	0.00	0.15	0.23	0.31	0.39
EM	2900.78	2901.55	2900.88	2901.85	2904.83

Description: Calculated based on Tables 1 and 2.

Table 4: Cellulase enzyme activity, crude fiber, and fiber digestibility of *Azolla microphylla* fermented with several types of fungi.

Treatment (Types of fungi)	Cellulase activity (U/ml)	Crude fiber decrease (%)	Fiber digestibility (%)
A	1.50± 0.03[a]	41.95± 0.95[a]	50.83±1.35[a]
B	1.56± 0.02[a]	44.83± 0.93[a]	54.86±1.30[a]
C	1.22± 0.04[b]	29.09± 0.90[b]	42.58±1.35[b]

Note: Different superscripts in the same column show very significant differences (P<0,01); A= *Lentinus edodes;* B= *Pleurotus ostreatus;* C= *Phanerochaete chrysosporium.*

The effect of fungi on protein increase and nitrogen retention of *A. microphylla* is seen in Table 5.

Table 5: Increased protein and nitrogen retention of *Azolla microphylla* fermented with several types of fungi.

Treatment (Types of fungi)	Increased protein (%)	Nitrogen retention (%)
A	45.99± 0.35[a]	65.33± 0.46[a]
B	46.89± 0.31[a]	66.50± 0.45[a]
C	28.17± 0.37[b]	55.21± 0.47[b]

Note: Different superscripts in the same column show very significant differences (P<0,01). A= *Lentinus edodes;* B = *Pleurotus ostreatus;* C= *Phanerochaete chrysosporium.*

The results of the statistical analysis showed that the type of fungi affected was highly significant (P<0.01) on increased protein and nitrogen retention from *A. microphylla* fermented. The result of the DMRT test showed that the increased protein and nitrogen retention of fungi *Pleurotus ostreatus* (treatment A) and *Lentinus edodes* (treatment B) are significantly higher (p<0.01) than *Phanerochaete chrysosporium* (treatment C).

Phase 2 effect of treatment on performance of broilers

The influence of the *Azolla microphylla* fermented with *Lentinus edodes* in feeds on feed consumption, body weight gain, and feed conversion can be seen in Table 6.

Table 6: Feed consumption, body weight gain, and feed conversion of broilers affected by the use of AMF in the feed.

Treatment	Feed consumption (g/head)	Body weight gain (g/head)	Feed conversion
A (0% AMF)	2057.00±12.21[a] 10.468.980.45[a]	1157.38±11.22[a]	1.78±0.01[a]
B (10% AMF)	2044.06±10.41[a]	1123.56±10.23[a]	1.82±0.04[a]
C (15% AMF)	2036.19±9.21[a]	1117.00±10.28[a]	1.82±0.04[a]
D (20% AMF)	1998.44±10.65[a]	1114.50±10.41[a]	1.79±0.02[a]
E (25% AMF)	1770.88±10.29[b]	1054.56±11.21[a]	1.68±0.03[b]

Information: Different superscripts in the same column show a very significant difference (P<0.01).

The results of the statistical analysis showed that the *Azolla microphylla* fermented affected highly significantly (P< 0.01) in feed consumption, body weight gain, and feed conversion of broilers from *A. microphylla* fermented. The result of the DMRT test showed that utilization 25% AMF is significantly lowest (p<0.01) on feed consumption, body weight gain, and feed conversion.

The influence of *Azolla microphylla* fermented with *Lentinus edodes* in the diet on the percentage of the carcass, the rate of abdominal fat, and the taste of carcass meat showed in Table 7.

Table 7: Percentage of the carcass, percentage of abdominal fat, and taste carcass meat of broiler fed *Azolla microphylla* fermented products with *Lentinus edodes* in the diet.

Treatment	Percentage of the carcass	Abdominal fat percentage	Carcass meat taste
A (0% AMF)	61.51±0.02[a]	1.98±0.04[a]	3.05±0.03[c]
B (10% AMF)	61.45±0.01[a]	1.85±0.01[a]	3.13±0.01[c]
C (15% AMF)	61.34±0.04[a]	1.81±0.02[a]	3.35±0.04[b]
D (20% AMF)	61.29±0.03[a]	1.75±0.03[a]	4.15±0.02[a]
E (25% AMF)	60.64±0.04[b]	1.73±0.04[b]	4.28±0.04[a]

Information: Different superscripts in the same column show a very significant difference (P<0.01).

The results of the statistic analysis showed that the *Azolla microphylla* fermented affected highly significantly (P< 0.01) on the percentage of the carcass, percentage of abdominal fat, and taste of carcass meat of broilers from *A. microphylla* fermented. The result of the DMRT test showed that utilization of 25% AMF is significantly lowest (p<0.01) on the percentage of the carcass but highest (P<0.01) on the rate of carcass meat taste.

Phase 1 determination of the best types of lignocellulolytic fungi on the nutrient quality of fermented *Azolla microphylla*

The activity of cellulase enzymes is higher in treatment B and treatment A than treatment C. It is caused both treatments are in the fast-growth phase or exponential phase at nine days fermentation. Fertile growth of the fungi *Pleurotus ostreatus* and *Lentinus edodes* can be observed visually in the presence of many fungal mycelia, white and evenly distributed on the substrate. The fertile growth of *Pleurotus ostreatus* and *Lentinus edodes* is also due to the room temperature during fermentation (22-24˚C) which is at the optimal temperature for these two fungi. According to Abou-Taleb (2009), several factors that influence cellulase enzyme activity include pH, temperature, substrate consented, incubation time, and the presence of cofactors and inhibitors. According to Valentine et al. (2017), the optimal temperature for *Lentinus edodes* is 24 ˚C, and the optimal temperature for *Pleurotus ostreatus* growth is 22-28˚C. The development of fertile *Pleurotus ostreatus* and *Lentinus edodes* results in increased cellulase enzyme activity, breaking down cellulose into glucose. Both of these fungi can degrade lignin and cellulose because they produce ligninase and cellulase enzymes. According to Rao (2009), cellulase enzymes can degrade cellulose through a catalytic process to release sugar (glucose).

Found the lowest cellulase enzyme activity was in treatment C (fermentation with *Phanerochaete chrysosporium*. It is related to the infertile and uneven fungi growth due to low cellulase enzyme activity. The unproductive increase of *Phanerochaete chrysosporium* is caused by low room temperature during fermentation, namely 22-24˚C (rainy season). According to Rulianah et al. (2017), the cellulase enzyme produced by *Phanerochaete chrysosporium* has the highest activity between temperatures of 25˚C-40˚C. The action of cellulase enzymes produced by *Phanerochaete chrysosporium* as cellulose-degrading will increase with increasing temperature to the optimal temperature limit.

The fiber content of *Azolla microphylla* and rice bran before fermentation was 29.83% dry matter with a lignin content of 22.64% and cellulose by 17.36%. There was a decrease in fiber after fermentation which can see in Table 4. The high reduction of fiber in treatment B (fermentation with *Pleurotus ostreatus*) was caused by cellulase and laccase activity), which also increased, namely 1.26 U/ml and 14.92 U/ml in treatment B so that the cellulose content and lignin decrease due to low crude fiber content. In treatment B, the cellulose content decreased from 17.36% to 10.08%, and the lignin content decreased from 22.64% to 11.44%; thus, the crude fiber content was low. According to Alarcon *et al*. (2003), the low range of fiber after fermentation is due to extracellular enzymes produced by the fungi *Pleurotus ostreatus* which result in degradation of the fiber cell wall components. According to Belitz et al. (2008), *Pleurotus ostreatus* produces cellulase enzymes that break cellulose into glucose. *Pleurotus ostreatus* also produces extracellular ligninase enzymes. Ligninase enzymes consist of lignin peroxidase (LiP), Manganese peroxidase (MnP), and laccase.

The decrease in crude fiber in treatment A (fermentation with *Lentinus edodes* related to the cellulase enzyme and laccase enzyme. Cellulase enzyme and laccase enzyme in treatment A 1.20 U/ml and 13.89 U/ml, respectively. The high cellulase activity causes the cellulose and lignin content to decrease, due to which the crude fiber content is low. The cellulase activity caused the cellulose content to drop from 17.36% to 10.27% and the lignin content to drop from 22.64% to 12.03%; as a result, the crude fiber content was low. According to Santos et al. (2012), the cellulase enzyme can degrade cellulose through a catalytic process. According to Elisashvili et al. (2007) and Nuraini et al. (2017), *Lentinus edodes* can produce the cellulase enzyme to degrade cellulose into glucose. According to Denny and Sutapa (2013), the decrease in crude fiber is also caused by the ligninase enzyme produced by *Lentinus edodes* which can degrade lignin. According to Grzegorz et al. (2017), *Lentinus edodes* degrade lignin because it has lignin peroxidase, manganese peroxidase, and laccase.

The low decrease in crude fiber in treatment C (fermentation with *Phanerochaete chrysosporium*) was due to the cellulase enzyme activity of 0.82 U/ml and the laccase enzyme 9 U/ml. The low activity of cellulase and laccase enzymes causes only a small amount of cellulose and lignin to be changed so that the decrease in crude fiber is common.

The high digestibility of crude fiber in treatment B (fermentation with *Pleurotus ostreatus*) and treatment A (fermentation with *Lentinus edodes*) was associated with a high fiber decrease in treatment B and A. The fiber content in feed ingredients is closely related to the level of digestibility. According to Lan et al. (2005), the high crude fiber content in the feed will interfere with the efficiency of using other food substances, causing the digestibility level to decrease. According to Singh and Kim (2021), fermentation also affects crude fiber digestibility. Fermented food substances usually have better nutritional

value than the original material because catabolic microorganisms will break down complex components into simpler substances that are easier to digest.

The digestibility of crude fiber in treatment B (fermentation with *Pleurotus ostreatus*) and treatment A (fermentation with *Lentinus edodes*) is high. It was also due to the increased activity of the cellulase enzyme and laccase enzyme in both treatments. The cellulase and laccase enzymes loosened the lignocellulose bonds (lignin and cellulose bonds). The cellulase enzyme can break down cellulose into glucose. The ligninase enzyme can break down lignin into phenolic compounds, CO2, and H2O to increase digestibility. According to Singh et al. (2014), digestibility has a negative correlation with crude fiber. The fiber content is lower, the digestibility of the fiber higher.

The digestibility of crude fiber in treatment C (fermentation with *Phanerochaete chrysosporium*) is low. Due to the cellulase enzyme 0.82 U/ml and the laccase enzyme 9 U/ml. They are causing an insufficient decrease in crude fiber 29.09% in treatment C. According to Prawitasari et al. (2012), the high fiber content in the feed causes the low digestibility of fiber. Fiber digestibility is influenced by fiber content. High fiber content results in at least the stored material range and is not appropriately utilized to excreted through excreta. Wahju (2004), fiber can only be digested a little by monogastric animals. The high fiber in the feed will reduce the efficiency of using other food substances; besides that, the effect of fiber that cannot digest comes out again through feces so that the poultry production and growing imperfect. Fiber digestibility is low because broilers do not have cellulase enzymes (Djulardi et al., 2018). According to Maynard et al. (2005), the factors influencing fiber digestibility include fiber content of the feed, microorganisms activity, and fiber consumption. In this *Azolla microphylla* study, the best results were fiber digestibility in treatment B (fermentation with *Pleurotus ostreatus*), namely 54.86%, and in treatment A (fermentation with *Lentinus edodes*), which was 50.83%. According to Nuraini et al. (2017), fermentation with *Lentinus edodes* on palm oil sludge substrate obtained fiber digestibility of 60.95%.

The high crude protein in treatment A (fermentation with *Lentinus edodes*) is the mycelium that grows fertile and evenly, causing increased microbial growth. There is an opportunity for microbes to contribute high enough protein. Widiyastuti dan Hidayat (2017), the increase in protein is related to protein from microbial cells, which increases during fermentation. According to Noferdiman et al. (2008), the enzymes produced by microbes are proteins. Fungi *Lentinus edodes* produce cellulase, ligninase, and xylanase enzymes (Elisashvli et al., 2008; Nuraini et al., 2017) and protease (Souza et al., 2016). According to Garraway and Evans (2009), fungal cell walls contain 6.3% protein, while cell membranes in hyphae fungi contain 25.45% protein and 25.30% carbohydrates. According to Cope (2018), crude protein consists of pure protein and Non-Protein Nitrogen (NPN). The purified protein is meant by pure protein composed of amino acids linked by peptide bonds. NPN is a non-protein compound that contains nitrogen such as free amino acids, nucleic acids, amino acids, urea, nitrates, etc. NPN in this fermentation product can be in the form of nucleic acids donated from the microbial body. Therefore, crude protein increases from microbes enzymes and the microbial body nucleic acids.

The high crude protein in treatment B (fermentation with *Pleurotus ostreatus*) grows fertile and evenly and white on the substrate. During the fermentation period of 9 days, *Pleurotus ostreatus* was in a fast growth phase or exponential phase so that many mycelia grew. Besides, the fertile growth of fungi is influenced by the availability of nutrients in substrates suitable for the development of *Pleurotus ostreatus*. The increase in protein content is in line with *Pleurotus ostreatus* mycelium growth because the mycelium body consists of nitrogen-containing elements. The high protein is also caused by the protease enzyme *Pleurotus ostreatus,* which breaks down protein into amino acids. Also, the increase in protein is related to the fermentation process to produce enzymes, and the enzyme itself is a protein (Noferdiman et al., 2008).

The low crude protein in treatment C (fermentation with *Phanerochaete chrysosporium*) was due to mycelium infertile and uneven growth, causing microorganisms to grow biomass slightly a little, which caused a small contribution of protein from microbes so that crude protein was low. This fungus does not produce protease enzymes. Protein breakdown is not as high as in treatments A and B. *Phanerochaete chrysosporium* is a white-rot fungus known for degrading lignin. Fermentation using *Phanerochaete chrysosporium* as a solid substrate allows the complex materials components to digest into easier. The high digestibility of crude fiber increases nutritional value and metabolic energy (Shrestha et al., 2008).

The high nitrogen retention in treatment A (fermentation with *Lentinus edodes*) was also associated with increased protein consumption, namely 4.36 g / head. The crude protein content in fermentation products is high; the more protein consumed, digested, and absorbed by the livestock body, the lot N (nitrogen) is left in the body. One factor affecting nitrogen retention is the consumption of feeds, especially protein and amino acid consumption. According to Wahju (2004), nitrogen retention is influenced by several factors, including food digestibility in the feed. If the protein quality is high or one of the amino acids is high, the nitrogen retention is also high.

The high nitrogen retention in treatment B (fermentation with *Pleurotus ostreatus*) was in line with the high crude protein content consumed by livestock, namely 4.41 g/head. According to (Noferdiman et al., 2008), increased protein consumption will result in more protein being digested. More of it is left in the body, resulting in nitrogen retention increases. Feed consumption factors that affect nitrogen retention, especially protein consumption. If nitrogen retention is high, the protein quality is high (Maynard et al., 2005). The high nitrogen retention in treatment B (fermentation with *Pleurotus ostreatus*) suggests that the protein quality of *Azolla microphylla* products fermented with *Pleurotus ostreatus* is high.

The low nitrogen retention in treatment C was 55.21%, along with the low protein consumption, namely 3.77 g/head, and crude protein, 26.21%. The low crude protein content in fermentation products means that the protein consumed by livestock is also common, resulting in less nitrogen left in the body. An increase influences nitrogen retention in protein levels in the feed. If the protein quality is low or one of the amino acids lacks, nitrogen retention will be low (Maynard et al., 2005). Nitrogen retention is the food protein left in the animal body or the difference between the amount eaten and absorbed by the animal body and excreted through feces and urine. Nitrogen retention is a method for assessing feed protein quality by measuring nitrogen consumption and nitrogen excretion in feces and urine to see how much nitrogen is left in the body (Wahju, 2004).

Phase 2 effect of treatment on performance of broilers

The feed consumption in treatment B (10% AMF), C (15% AMF), and D (20% AMF), similar to feed consumption in treatment A (0% AMF), showed that the AMF with *Lentinus edodes* is palatable to the level of 20% in broiler feeds and has good palatability. Treatment D (20% AMF) with *Lentinus edodes* contained less corn and soybean meal than treatment A, although there was a reduction of 13.92% corn and 53.33% soybean meal. Treatment D has the same feed quality as treatment A, although the content of corn and meal is less than treatment A. According to Nuraini et al. (2017), feed ingredients after fermentation produces good physical quality and high palatability compared to those not fermented. *Azolla microphylla,* fermented with *Lentinus edodes*, has a better flavor, increasing palatability. According to Hidayat et al. (2006) and Nuraini et al. (2020), the fermentation process can provide beneficial physical and chemical changes such as aroma, taste, texture, and better digestibility than the original material. According to Murugesan et al. (2005), the fermented product has a preferred flavor and contains several vitamins (B1, B2, and B12) so that it is preferred when compared to the original material.

The feed consumption in treatment E is low. Due to the high crude fiber in treatment E, which was 7%, causing the feeds consumed not to be adequately digested by broilers and causing nutrient absorption to be hampered because poultry had limitations in digesting crude fiber. According to Amrullah (2004), high crude fiber causes poultry to feel full, reducing consumption because crude fiber is voluminous. An excellent crude fiber in the diet is recommended at most 6.7%. The higher the crude fiber content in the feed, the slower digestion and nutrient absorption rate. According to Permana (2012), the increased crude fiber in the feed can reduce digestibility, absorb nutrients, and reduce the availability of nutrients for growth.

The low consumption of feeds in treatment E was also due to the darker color of the feeds. The fermentation of *Azolla microphylla* causes the fermented product to be darker than the original color. The initial flush of *Azolla microphylla* is green, after being fermented, the color of this fermented product becomes darker, namely brown. The color of the diet in treatment E (25% AMF) was darker than the color of the diet of treatment A (0% AMF), so the feed of treatment E was less favorable because of the dark color. This follows the opinion of Rasyaf (2008), which states that broilers prefer light and bright-colored feeds.

Azolla microphylla fermented with *Lentinus edodes* to a level of 20% which reduced the use of corn and soybean meal in the feed had the same quality as the feed in treatment A (a feed that used a lot of corn and soybean meal), and this could be seen in the same increase in body weight. This also shows that the quality of AMF products has improved due to the help of cellulase and ligninase enzymes that degrade crude fiber. For maximum body weight gain, it is necessary to pay attention to the feed quality. The feed must contain nutrients in sufficient and balanced conditions to support maximum growth (Rasyaf, 2008). The nutrient content in the diet and feed intake is affected body weight (Chiang et al., 2010; Jahan et al., 2006). The quality of the feed needs to be considered to obtain maximum body weight gain.

The same increase in body weight in treatments A, B, C, and D was caused by consuming the same feed in the four treatments, especially protein consumption. Protein consumption in treatment A of 15.43 g/head/day, treatment B 15.33 g/head/day, treatment C 15.27 g/head/day, and treatment D 14.99 g/head/day. According to Chiang et al. (2010), feeds and food substances can influence high and low body weight gain. Added that the consumption of feeds would be related to body weight gain; the higher the consumption of feeds, the higher body weight gain, and vice versa.

The lowest body weight gain in treatment E with the

addition of *Azolla microphylla* fermentation, as much as 25%, is due to the consumption of feeds in treatment E being also low. The low consumption of feeds was due to the high crude fiber contained in the E treatment feeds, so the feeds consumed were not utilized by broilers properly, thus causing a decrease in body weight gain. According to Anita et al. (2012), the high crude fiber in the feed will reduce feed consumption and digestibility of food substances because crude fiber is difficult to digest by poultry and will be wasted with feces because the body does not absorb it. The low consumption of feeds also reduces the number of nutrients that enter the broiler body. Therefore, the body weight gain is also low.

The feed conversion is used to see the efficiency of the use of feed by livestock, or it can be said as the efficiency of converting feed into the final product in the form of meat. There was no significant difference in the treatment of feed conversion due to feed consumption being directly proportional to body weight gain from treatment A to E. The resulting feed conversion was also the same. The higher the feed consumption in each treatment, the higher the resulting body weight gain. The size of the feed conversion is determined by the amount of feed consumption and body weight gain because the feed conversion is obtained from feed consumption divided by body weight gain. Rasyaf's (2008) opinion that feeds conversion compares the amount of feed consumption in one week with the increase in the body weight of chickens achieved that week.

The carcass portion in treatment B, C, and D was not significantly different from treatment A because the live weights that are also different are not significant. Carcass percentage was obtained from carcass weight divided by live weight multiplied by 100%. According to Soeparno (2005), the carcass percentages were not significantly different because the final body weight was in line with the carcass weight. The proportion of body parts or the carcass produced was the same. One factor that affects the percentage of broiler carcasses is live weight. The same live weight in treatments B, C, and D with treatment A was associated with the same feed consumption in treatments B, C, and D.

The difference in the percentage of carcasses in treatment B, C, and D with treatment A was not significantly different, indicating that the feed quality was the same between treatments B, C, and D with treatment A. The quality of protein in treatment D with 20% AMF could match the protein quality in treatment A although, in treatment D, there was a reduction of 13.92% of corn and a reduction of 53.33% soybean meal. Fermentation carried out with *Lentinus edodes* produces a flavor that can increase the palatability of AMF products. As a result, the feed given to broilers is preferred or palatable. According to Chojnacka (2010), the fermentation material produces good quality and high palatability compared to the unfermented. According to Murugesan et al. (2005), the fermented product has a preferred flavor and contains several vitamins (B1, B2, and B12) so that it is preferred when compared to the original material.

The low percentage of broiler carcasses in treatment E using 25% AMF with *Lentinus edodes*, which was 60.64%, was caused by the higher crude fiber content in the feed (rough fiber in the feed 7.00%). The high crude fiber content in the feed-in treatment E can reduce digestibility. The high crude fiber in the feed can cause broilers to feel full because crude fiber is bulky (satisfying), so the amount of food and nutrients that enter is small, resulting in low chicken weight and a low percentage of broiler carcass. According to Amrullah (2004), high crude fiber causes poultry to feel full, reducing consumption because crude fiber is voluminous. According to Permana (2012), the increased crude fiber in the feed can reduce digestibility, absorb nutrients, and reduce the availability of nutrients for growth. Santoso (2008), crude fiber for good broilers is recommended at 6.7%. According to Permana (2012), it was further explained that the ability of the bacteria in the cecum to carry out fermentative digestion is not yet known.

The carcasses in treatment E are low, namely 25% AMF with *Lentinus edodes* in the feed. This is due to the low live weight in treatment E. The carcass percentage is obtained from the ratio of carcass weight to live weight multiplied by 100%; according to Amrullah (2004), the achievement of carcass weight is closely related to the living weight.

The low percentage of carcasses in treatment E, namely the use of 25% AMF with *Lentinus edodes* in the feed, also caused by feed consumption (feed consumption) 1770.88 g/head) broiler in treatment E was also low. Low feed consumption was associated with a darker feed color in treatment E. Fermentation of *Azolla microphylla* with *Lentinus edodes* causes discoloration. At first, *Azolla microphylla* is green, but after fermentation, it is white, and after drying, it turns brown. In treatment E, the use of the product AMF was 25%, and the feeds of treatment E were darker in color than the feeds of treatment A (AMF 0%), B (AMF 10%), C (15% AMF), and D (20% AMF). Broilers prefer lighter-colored feeds to dark-colored feeds. In treatment E, the dark color of the feed resulted in the consumption of broiler feeds in the treatment with the use of 25% lower. According to Rasyaf (2008), broilers prefer light and bright colored feeds.

The difference between the treatment of abdominal fat percentage indicates no energy accumulation in the given

feed. Feed prepared based on iso-protein and iso-energy with a crude protein content of 21,3% and metabolic energy of 2900 kcal/kg as recommended by Scott et al. (1982). According to Rasyaf (2008), the accumulation of fat in a chickens' body, including abdominal fat, occurs because the energy, which is the result of the metabolic process of nutrients entering the chicken's body, exceeds the level of needs required. Both for basic life and production. The metabolic energy content is directly proportional to the percentage of abdominal fat.

Abdominal fat percentage broiler strain CP-707 obtained in treatment D (20% AMF) for five weeks of the study was 1.75%. Haro (2005) reported that the body fat content of broiler chickens reached 13-14.5% of live weight, while the percentage of abdominal fat in the body of chickens reached 2-3% of live weight. This value is also lower than the percentage of abdominal fat in broilers aged five weeks obtained from Massolo (2016), namely by 2.15%. Lu et al. (2007) reported that broilers reared in a warmer environment showed abdominal fat weightless men. Abdominal fat accumulation in broiler chickens can reduce energy consumption (Rosa et al., 2007).

The high score of broiler carcass meat tastes in treatment D using 20% AMF with *Lentinus edodes,* namely 4.15, and treatment E using 25% AMF with *Lentinus edodes,* i.e., 4.28. Due to the high content of glutamic amino acid in the feed-in treatment D, which is 0.31%, and in treatment E, which is 0.39%. *Azolla microphylla* fermented with *Lentinus edodes* contains 1.54% of the amino acid glutamate (Nuraini and Mirzah, 2021). The glutamate content in mushrooms is the essential ingredient for flavoring food because it causes a savory taste. The presence of glutamate in mushrooms can give a delicious flavor to food, even almost the same as meat (Widyastuti et al., 2015). According to Renee (2015), *Lentinus edodes* or Shiitake mushrooms have the highest glutamic amino acid content, 2.58 g/100 g.

CONCLUSIONS AND RECOMMENDATIONS

The study concluded that the fermentation of *Azolla microphylla* with *Lentinus edodes* and *Pleurotus ostreatus* is the best treatment. *Azolla microphylla* fermented with Shiitake mushroom (*Lentinus edodes*) can be used up to 20% in the feed and maintain the broiler performance.

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NOVELTY STATEMENT

Research on Improving Azolla microphylla through Fermentation with Lignocellulolytic Fungi and its Application in Broiler Feed has never been done by previous researchers.This study succeeded in finding a suitable alternative feed and ration formulation for broiler with 20% AMF in the diet.

AUTHOR'S CONTRIBUTION

Nuraini created the idea, designing the experiment (fermentation and utilization AMF to broiler), analyzing data, and writing this article. Mirzah contributed to the utilization of AMF to broiler and checked the written paper. Harnentis contributed to fermentation and assisted in the revision of the article.

Conflict of interest

The authors have declared no conflict of interest.

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