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Replacement of soybean meal with leucaena leaf meal fermented by proteolytic bacteria in diets of nile tilapia (*Oreochromis niloticus*)

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Abstract
The use of alternative feeds aims to increase productivity and reduce the feed costs in aquaculture production. This objective of this study was to determine the nutritional value and optimal rate of fermented Leucaena Leaf Meal (LLMF) as well as the replacement of soybean meal (SBM) by LLMF in diets for Nile tilapia (*Oreochromis niloticus*). Five different iso-proteinous diets were prepared and fed to three replicates trial of group tilapia. Fish were fed 28% protein diets with substitution of LLMF 0%, 25%, 50%, 75%, and 100%. The parameters that have been observed consist of the survival rate (SR), specific growth rate (SGR) and feed efficiency (FE). The results showed that the best results of SR, SGR and FE were observed in LLMF 0% group; 100%, 1.27%, and 31.47%. However, these results were not significant differences (*P>0.05*) from LLMF 25% group; 93.33%, 1.19%, and 28.25%. The experimental study from the present work suggested that LLMF can be used to substitute until a 25% level which can provide the survival rate, specific growth rate and feed efficiency as well as feeds without LLMF.

Keywords: Leucaena, fermentation, substitution, diet, tilapia

1. Introduction
Soybean meal (SBM) is currently is the most common plant-based protein for commercial fish culture due to its high protein content, balanced amino acid profile, consistent quality and abundant supply [1]. Due to its high demand as a protein-based for diets, the SBM is a competitive ingredient and its cost increased significantly [2]. In order to reduce feed costs, alternative resources from the plant are considered as an economical and environmentally friendly for feed ingredients.

Leucaena leaf meal (LLM) has a high enough protein content (of approximately 25%), good amino acid composition and a high β-carotene [3]. However, the utilization of Leucaena has been limited due to the presence of a toxic non- protein amino acid or anti-nutritional factor called mimosine [4]. The effects of the anti-nutritional factor mimosine have been demonstrated in rats [5] and fish [6]. In addition, its presence a negative impact on decreasing the growth performance if consumed intensively [7]. It has been reported that mimosine can be reduced to a relatively less toxic form through various methods of processing such as by sun drying, soaking, boiling [6, 8] and inoculating with specific strains of gut bacteria [8].

One of the microbes used in the processing ways was lactic-acid bacteria [3, 8]. Lactid-acid bacteria is a proteolytic microbe that has been proven to secrete protease enzymes and increase the digestibility of feed protein sources [3]. One way to improve the quality of LLM is to utilize the services of proteolytic bacteria.

The use of 20% Leucaena leaf which soaked and dried for three days as a substitute for fish meal gave the best specific growth rate for Catfish (0.31%) [9]. Substitution of Leucaena leaf at the level of 25% for *Oreochromis niloticus* diets after being soaked in the water for 48 hours obtained a daily growth rate of 1.8% [6]. Similar findings were reported by Falaye [10] observed that substitution of peanut meal by LLM for the diet of tilapia fingerlings could be used up to 25% level after 48 hours of soaking and 24 hours drying.

The present study aimed to investigate the nutritional value of LLM as a substitutional meal for Nile tilapia after being dried, soaked, and fermented by proteolytic bacteria in order to improve the quality of LLM and provide the best survival rate and growth rate of Nile tilapia.

2. Materials and Methods

2.1 Processing of Leucaena leaf powder

Leucaena leaves were collected manually from CIFOR, Bogor, Indonesia. Leaves were thoroughly removed from the branches, rinsed with water, and then soaked in freshwater for 72 hours, sun-drying 48 hours, and ground by hammer mill became Leucaena leaf powder at Research Institute of Freshwater Aquaculture, Bogor, Indonesia.

2.2 Fermentation process

Fermentation of Leucaena leaf using the lactic-acid bacteria, namely the proteolytic bacteria which inoculated from goat rumen fluid and was determined as *Lactobacillus* spp. with 10^8cfu ml^{-1} of bacteria suspension. At pre-experimental stage, those bacteria were tested for proteolytic activity into the LLM with three-level treatments of the biomass of LLM, namely, 25ml $100g^{-1}$, 50 ml $100g^{-1}$, and 75 ml $100g^{-1}$. The best result with the highest protein level was $50ml/100g^{-1}$. Afterward, a sample of 2 Kg of finely fermented LLM was used for the experimental diets namely Leucaena Leaf Meal Fermented (LLMF).

2.3 Experimental animals and conditions

The experimental fish, Nile tilapia *Oreochromis niloticus*, were obtained from a farmer at The Parung fish market, Bogor, Indonesia. They were acclimatized for seven days in polyethylene cylinder tank of Research Institute of Freshwater Aquaculture, Bogor, Indonesia and they were fed with commercial diets. After being kept in a polyethylene cylinder tank, they were moved and maintained into the aquarium in the indoor room. They were divided into five types of experimental treatments with three types of replication. At the first step of the experiment, the initial body weight was weighted and the initial length was measured, and the mean wet weights were different between each treatment. The average body weight was 11.00 ± 0.25 g and the length ranged 8-9 cm. Each trial group was separately reared in a 50cm x 50cm x 30cm square aquarium for about fifty days. Each tank reared 10 fish and each group was supplied with 10 l min^{-1} of filtered and aerated water in a circulation system. Every 10 days the total biomass of each tank was weighted for sampling, the supplemental fish were prepared to change the dyed fish during the experimental periods. Temperature, pH, and DO were measured during the experiment. At the end of the experiment, fish were weighted to obtain the final body weight.

2.4 Preparation of the experimental diets

Five iso-nitrogenous (Approximately 28% Crude Protein and 6% Lipid) experimental diets were prepared with different levels of Leucaena leaf meal fermented (LLMF) after through the fermentation process as mentioned above; including a diet without LLMF as a control. LLMF was added to the formulated experimental diets at 0% (LLMF0), 25% (LLMF25), 50% (LLMF50), 75% (LLMF75) and 100% (LLMF100) replacement level of Soybean meal (SBM) as a protein source. Each experimental diet was mixed and dried as 3 mm pellets at 60 °C and then subsequently stored at 4 °C until use. Each experimental diet was randomly assigned to three different replication tanks and the rearing period was 45 days. Fish were fed three times a day with a feeding rate of 2-3% of BW for the appropriate feeding rate for Nile tilapia.

2.5 Proximate analyses

The proximate analyses of the raw materials of LLM, LLMF, and experimental diets were carried out following the procedures described in Official Methods of Analysis of AOAC International 17th edition, AOAC 2003. Chemical compositions were analyzed at the Nutrition Laboratory of Faculty of Animal Husbandry, Padjadjaran University. The analysis of moisture was measured by drying diets to show a constant weight in an oven at 105 ^{0}C. The analysis of total crude protein was carried out by Kjeldahl method. The analysis of lipid content were carried out by The Soxhlet method using extraction with petroleum ether. The analysis of crude fiber were measured by an automatic analyzer. Total ash content was measured by mineralization of the sample at temperature 600 ^{0}C for 2 hours.

2.6 Data analysis

The growth performance of experimental fish was evaluated by determining the survival rate (SR), Feeding Efficiency (FE), and Specific Growth Rate (SGR). Fish were selected from each experimental tank and measured after rearing periods. The formulae used are as follows:

SR (%) = (Total number of alive fish/total number of fish) × 100

FE (%) = [100× (final weight–initial weight) × (initial number of fish + final number of fish)]/ dry weight of feed delivered

SGR (%) = 100 × [ln (final weight) – ln (initial weight)] /days

Prior to statistical analyses, all growth data were analyzed by the ANOVA test to determine if each parameter was significantly different and using SPSS software.

3. Results

3.1 Proximate analyses of LLM

The proximate analyses of LLM showed that it contained 11.77% crude protein, 0.21% crude lipid, and 20.33% crude fiber (Table 1).

Table 1: Proximate analyses of LLM and LLMF ingredient used in the experimental diet (Dry weight basis)

Chemical composition	Present Study		Falaye *et al* (2002)	Bairagi *et al* (2004)	
	LLM	LLMF	LLM*	LLM	LLMF**
Crude Protein	27.58	28.12	24.05	20.35	29.77
Crude Lipid	3.08	3.88	2.05	5.35	5.02
Crude Fiber	38.02	36.96	20.20	6.15	5.92
Nitrogen-free Extract	25.66	23.88	35.20	52.35	N/A
Moisture	1.20	2.10	7.50	7.35	N/A
Ash	4.46	5.06	11.00	8.45	N/A

*LLM were processed by soaking in water for 48 hours and sun drying for about 24 hours

**LLM was inoculated (treated) with *Bacillus subtilis* and *Bacillus circulans* culture separately at the rate of108 bacterial cells per gram of dried LLM

The nutritive value of the experimental feeds is shown in Table 2. The total crude protein in each treatment was similar (28.16-28.30%), and total crude lipid ranged 60.1-62.5 g kg-1 DM. Diet palatability was assessed by direct observation of

fish behavior and responses to the diets.

Table 2: The composition of dietary ingredients in the experimental diets (g kg-1 dry matter)

Ingredients	LLM0	LLM25	LLM50	LLM75	LLM100
Fish meal	10.0	10.0	10.0	10.0	10.0
SBM*	34.0	25.5	17.0	8.5	0
LLMF**	0	8.5	17.0	25.5	34.0
Rice bran	21.0	21.0	21.0	21.0	21.0
Polard	20.0	20.0	20.0	20.0	20.0
Tapioca	10.0	7.5	5.5	3.5	1.0
Casein	0	2.5	4.5	6.5	9.0
Fish oil	2.0	2.0	2.0	2.0	2.0
Premix	2.0	2.0	2.0	2.0	2.0
CMC	1.0	1.0	1.0	1.0	1.0
Total	100	100	100	100	100

Chemical Composition					
Crude Protein	28.16	28.29	28.23	28.17	28.30
Crude Lipid	7.13	7.29	7.43	7.57	7.73
Nitrogen-free extract	49.19	45.63	42.65	39.67	36.11
Ash	8.69	8.44	8.20	7.97	7.72
Crude Fiber	3.83	7.16	10.13	13.09	16.42

Growth Performance

The results of the growth performance of Nile tilapia fed with various levels of LLMF are shown in Table 3. There was no mortality recorded during the 50 days feeding experiment. The highest SGR and FE were obtained from LLM0 in all rearing conditions, and SGR and FE in LLM100 were the lowest results. Specifically, SGR and FE of male Nile tilapia in the individual rearing condition treatment ranged 0.68-1.27%/day and 17.88-33.66%, respectively. SGR and FE of fish in LLM25 were significantly (p<0.05) greater than fish in LLM75 an LLM100 but not different from fish in LLM0 and LLM50.

Table 3: Growth performance of Nile tilapia fed diets supplemented with different levels of LLMF in different types of rearing condition[1]

Treatments	Survival rate (%)	Feed Efficiency (%)	Specific Growth Rate (%fish/day)
LLM0	100[a]	33.66±1.43[d]	1.27±0.05[d]
LLM25	100[a]	31.47±1.67[cd]	1.19±0.04[cd]
LLM50	100[a]	28.25±2.06[c]	1.07±0.07[c]
LLM75	100[a]	21.66±2.49[b]	0.81±0.10[b]
LLM100	100[a]	17.88±1.00[a]	0.68±0.04[a]

[1]Each value is the mean ± S.E.M. of data from replicate groups. Within a column, means with different letters indicate a significant difference (P<0.05).

The results of regression analysis determined the relationship between the effects of the substitution level of LLMF on the growth rate specifically showed a linear model with the equation: Y = 1.320 - 0.006x (Fig 1).

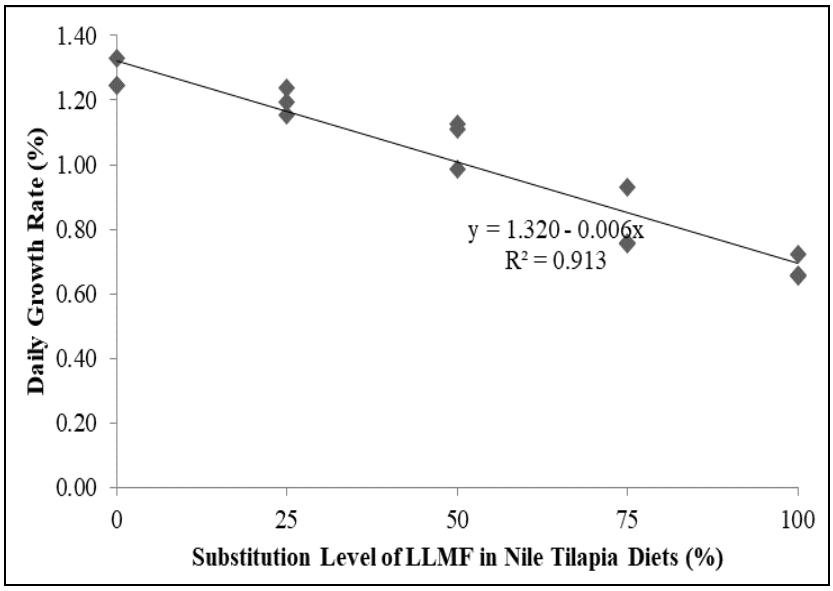

Fig 1: Regression analysis of daily growth rate (%) for Nile tilapia fed by various different level substitution of LLMF (CP 28%) during 50 days rearing periods.

Water quality parameters measured during the study were water temperature, pH, and DO. The measurement results showed that water quality during the experimental periods was in the range of the appropriate level for Tilapia culture (Table 4).

Table 4: Range of water quality analysis during the experimental periods in Nile tilapia rearing condition fed by various substitution level of LLMF.

Treatments	Water Temperature (^{0}C)	pH	DO (mg/L)
LLM0	27.8-28.1	6.93-6.96	3.64-3.71
LLM25	28.4-28.9	6.00-6.30	3.54-3.92
LLM50	27.6-28.9	6.79-7.05	3.29-4.22
LLM75	28.1-28.8	6.79-6.97	3.78-4.42
LLM100	27.7-28.6	6.90-6.94	3.74-4.42

4. Discussion

Based on the results presented in Table 1, the crude protein of LLM after fermentation (28.12%) was higher than before fermentation (27.58%). The result of the crude protein level

of LLM before fermentation in the present study was higher than those in previous studies about LLM diets, which contained 24.05% [10], and 20.35% CP [8]. Different results might be caused by the processes used in each research were different. After being fermented using proteolytic bacteria (*Lactobacillus* sp.), the percentage of CP in the present study was increased to 28.12%. In line with previous study [8], bacteria could help to increase the crude protein of LLM. However, the increasing level of crude protein of LLM fermented by *Lactobacillus* sp. in the present study was lower (0.54%) than LLM inoculated by *B. subtilis* and *B. circulans* (9.42%) [8].

Although the fermentation could decrease crude protein and decrease the crude fiber of LLM, the crude fiber contained in LLM of the present study was still quite high in the level of 38.02% before fermentation and decreased to 36.96% after fermentation. This result was quite higher than the previous research [10] with the soaking method was resulted in 20.20% of crude fiber and inoculating with *Bacillus* sp. showed 5.92% of crude fiber [8]. Differences in nutrient contents were thought to be due to the initial leaves used in each study according to the statement of [11] that the nutritive values of Leucaena leaf vary according to age, location, and species. The leaves used in this study contain high crude fiber so that even though fermentation has been done, it was still not enough to reduce the crude fiber. Even though before fermentation, LLM in our present study was sundried for 72 hours. In line with Agbo *et al*. [12] that the best time to sundried the LLM to increase the crude protein was 72 hours.

Our results showed that a 25% substitution of LLMF in diets can be added as the optimum result for SGR and FE for tilapia culture. From five experimental rearing conditions, the highest SGR values (P<0.05) were observed from treatment without LLM (LLM0) substitution (Table 3), although no significant differences with group fish in (P>0.05).

In our present study, the increasing addition of LLMF in diets decreased the growth performance because adding more LLMF might produce negative impact contributing to low growth rates. In accordance with previous studies which mentioned that the decrease in growth rate apparently due to the presence of anti-nutritional factors such as mimosine and tannins which could not fully being reduced and became the inhibitor of a protein, thus, the protein contained in LLM cannot be completely used for the growth [8, 10].

Studies about the utility of LLM in fish diets have been reported that LLM could be used for a fish feed up to 25% such as in African catfish, *Clarias gariepinus*, the inclusion levels of up to 30% but it is efficacious and cost-effective at 20% inclusion level [9]; in Nile tilapia, *O. niloticus*, the inclusion level of soaked LLM for 48 hours at level 25% gave the highest growth rate [6, 10].

5. Conclusion

In conclusion, the results also suggested by adding LLMF at the level of 25% of inclusion could give the best growth performance for Nile tilapia. However, because the utility of high levels of LLM in fish diets may lead to reduce growth performance, future research is necessary to investigate the optimum ways to omit the anti-nutritional factors in LLM and the maximum level of LLM that fish could accept as a component of diets. Thus, the utility of Leucaena leaf in fish diets could be provided a possible use for the unexploited resource of plant protein source.

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