Improving crude protein and crude fat content of Seligi leaf (*Phyllanthus buxifolius*) flour through probiotic fermentation

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Abstract. The increasing demand for fish feed causes the requirement for the raw materials of fish feed also increases. Therefore, alternative feed materials are urgently needed. This study aims to investigate Seligi leaf fermented with probiotics as an alternative for feed material. Two factors were investigated; fermentation time (5 and 7 days) and the dose of probiotics (0%, 2%, 4% and 6%). The two factors were arranged in a factorial Completely Randomized Design (CRD). Data were analyzed using analysis of variance, followed by Duncan's Multiple Range Test for any significant differences. The result showed that Seligi leaf flour fermented with 2% probiotic and fermented for 5 days had the highest crude protein, p<0.05, while reducing the crude fat content in Seligi leaf flour. The results also showed that there was no interaction between the fermentation time and probiotic dose with crude protein and crude fat content in Seligi leaf flour. This result suggests that the nutrition value of Seligi leaf flour as fish feed materials can be improved through probiotic fermentation.

1. Introduction

The feed is one of the most important factors which support the aquaculture business activities. The increase in the amount of demand for fish food causes the need for the raw materials of fish food to increase; therefore, alternative food materials are needed. Seligi leaf is a plant which is quite easy to obtain but is still not widely used properly because of its nutritional content which still needs to be improved to become a quality raw material for alternative fish food [1].

One way which can be done to improve feed ingredients’ quality is by fermentation using probiotics [2]. Previous studies suggested that probiotics could improve nutritional values of feed materials through several mechanisms including increasing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [3], increasing feed content. The fermentation process is affected by time length and dose [4]. This study aims to determine whether the addition of probiotics with different doses and time lengths on the fermentation process will affect the crude protein and crude fat content in Seligi leaf.
2. Material and methods

2.1. Materials
The present study used Seligi leaf, molasses, a consortium of probiotics, and aquades. Seligi leaf was freshly harvested from a farm of the Faculty of Veterinary Medicine, Universitas Airlangga. While, probiotic consortium (Bio MC-4) consisted of *Actinomyces* spp., *Bacillus* sp., *Enterobacter* sp., and *Cellulomonas* spp., [4] with the density of ~1.2 x 10⁹ CFU/ml [5].

2.2. Experimental design
The present study used a completely randomized design (CRD) with two factors. The first factor as the duration of fermentation consisting of two treatments (5 and 7 days) and the second factor was probiotic dose consisting of 4 treatments (0%, 2%, 4%, 6%). In total, there were 8 combinations of treatments, and each treatment had three replications therefore become 24 experimental units. Table 1 presented all combination of treatment used in the present study.

| Table 1. Combination of treatment factors in research |
|-----------------------------------------------|
| **Duration of Fermentation (H)** | **Probiotic Dose (P)** | **P0 (Control)** | **P1 (2%)** | **P2 (4%)** | **P3 (6%)** |
| H1 (5 days) | P0 | H1P0 | H1P1 | H1P2 | H1P3 |
| H2 (7 days) | P0 | H2P0 | H2P1 | H2P2 | H2P3 |

Note, P0 = 2 ml molasses + 0 ml probiotics + 28 ml aquadest; P1 = 2 ml molasses + 2 ml probiotics + 26 ml aquadest; P2 = 2 ml molasses + 4 ml probiotics + 24 ml aquadest; P3 = 2 ml molasses + 6 ml probiotics + 22 ml aquadest

The dose used was based on the results of the best dose analysis study conducted on the proximate analysis of papaya leaves fermentation using BioMC4 probiotics with a bacterial count of 1.2 x 10⁹ CFU/ml [6]. The dose of molasses used was 2%, which is under Ismi et al [7] which states that the administration of 2% molasses dose gives the best results in feed quality, while the water used is 30% of the total leaves flour.

2.3. Procedure
Seligi leaf was freshly harvested from a farm of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. The leaf was dried under the sun for three days to reduce water contents. The dried leaf was then ground using a grinding machine to produce Seligi-leaf flour. Thereafter, 100 gram of the Seligi leaves flour was weighed and put into a basin to be mixed and homogenized with the probiotic solution. The probiotic solution was prepared by diluting 2% molasses in 30% water, and probiotic concentration was adjusted according to the treatment in Table 1. The probiotic solution was then left for 24 hours in a closed container to get an anaerobic condition. 24 hours afterwards, the probiotic solution as put into a spray bottle and then sprayed to the Seligi leaf flour evenly, and placed into plastic bags and tied. The fermentation process was carried out for 5 and 7 days according to the treatment.

2.3.1. Parameter
The parameters observed in this study were nutrient content of crude protein, and crude fat) on fermented leaves of different duration and dose of probiotics. The value of the parameter content is known by doing a proximate analysis.

2.3.2. Data Analysis
Data analysis was performed by Analysis of Variance (ANOVA). If the result showed differences or significantly different then continued with the Duncan Multiple Range Test with a 95% confidence interval to find out which treatment gave the highest and lowest results [8].

3. Results and discussion

3.1. Crude protein
The results of the Analyze of Variance (ANOVA) test showed that there was no interaction between the length of duration of fermentation and the dose of crude protein content. Based on the results of statistical analysis, fermented Seligi leaves flour showed no significant difference (p > 0.05) between treatments, both on day 5 and day 7 of the crude protein content in the difference in fermentation duration. Statistical analysis (ANOVA) showed that there were significant differences (p < 0.05) in the use of different doses of crude protein content. A high crude protein value obtained at the use of probiotics at a dose of 2% (P1)
which is not different with the use of a dose of 4% (P2), but a dose of 4% (P2) is not different from the control (P0). Low crude protein values, obtained at the use of a dose of 6% (P3) which is not different from the treatment without the use of probiotics (P0). Based on the results showed that the dose of 2% probiotic gave the best result to increased crude protein content on Seligi leaves.

Table 2. The average of the crude protein content of Seligi leaves powder during treatment

| Probiotic Dose | Fermentation Time | Average |
|----------------|-------------------|---------|
|                | H1 (5 days)       | H2 (7 days) |         |
| P0 (0%)        | 13.24abc± 0.49    | 12.91bcd ± 0.20 | 13.07bc± 0.38 |
| P1 (2%)        | 13.75ab ± 0.58    | 13.43abc ± 0.15 | 13.59a± 0.42  |
| P2 (4%)        | 13.38ab ± 0.34    | 13.22abc± 0.43 | 13.30ab± 0.36 |
| P3 (6%)        | 12.65d ± 0.15     | 12.73cd ± 0.12 | 12.69d± 0.13  |
| Average        | 13.25abc ± 0.55   | 13.07a± 0.35   |

Note: Different superscripts in the same column and row show significantly different effects (p <0.05)

The improvement of crude protein content in fermented Seligi leaves flour is caused by a large number of microbes present in probiotics which can break down complex ingredients into amino acids [9], so these amino acids can be used by microbes to multiply themselves. According to Wuryantoro [10], the increasing number of microbial colonies during fermentation can indirectly increase the crude protein content because microbes are a source of single-cell protein.

Provision of molasses in the fermentation process can also help increase the content of crude protein because molasses is a source of carbohydrates containing amino acids which are often used as a source of energy for bacteria. Abun [11] states that in the anaerobic fermentation process added with molasses can increase crude protein due to anaerobic microbial activity that grows and develops by utilizing energy sources into microbial cells that are rich in protein content. Rachmasari [12] states that the high levels of carbohydrates (73.1%) and minerals (11.7%) in molasses are able to support the growth of bacteria so the crude protein can be increased.

Decreased crude protein on the 7th day of fermentation on probiotic doses of 4% and 6% caused by food sources that have been depleted then the bacteria no longer produce enzymes and death, but it can also be caused by too many doses, causing microbial competition which causes microbes to die and not produce enzymes in the fermentation process. Kanti [13] added that the nutritional value that is less than the amount of microbial biomass can result in a longer period of reduced biomass.

3.2. Crude Fat
The results of the Analyze of Variance (ANOVA) test showed that there was no interaction between the duration of fermentation and the dose of crude fat content.

Table 3. The average of the crude fat content of Seligi leaf powder during treatment

| Probiotic Doses | Fermentation Time | Average |
|-----------------|-------------------|---------|
|                 | H1 (5 days)       | H2 (7 days) |         |
| P0 (0%)         | 8.32abc± 0.73     | 9.32a± 0.22 | 8.82a± 0.73 |
| P1 (2%)         | 7.14c ± 0.35      | 7.96ab± 1.16 | 7.55b± 0.89 |
| P2 (4%)         | 7.41c ± 0.72      | 8.16ab± 0.50 | 7.79b± 0.69 |
| P3 (6%)         | 7.33c ± 0.33      | 8.59ab± 0.29 | 7.96b± 0.74 |
| Average         | 7.55c ± 0.68      | 8.51b± 0.78 |

Note: Different superscripts in the same column and row show significantly different effects (p <0.05)

Based on the results of statistical analysis, Seligi leaves flour fermented at different duration showed a significant difference (p <0.05) between treatments for crude fat content on day 5 and day 7. Lower crude fat values were obtained at the treatment 5 days compared to the value of crude fat on day 7. The results of statistical analysis showed that there were significant differences (p <0.05) in the use of different doses of crude fat content. The same low crude fat value is obtained when using probiotics at doses of 2% (P1) and 4% (P2). A high crude fat value is obtained at the use of a dose of 0% (P0) which is not different from the use of a 6% dose of probiotics (P3). The treatment of the use of probiotics 2% (P1) and 4% (P2) shows the results of the value of crude fat that is not different from the treatment of 6% (P3). Based on the results
obtained, the treatment of the use of 2% (P1) and 4% (P2) doses is an equally good treatment to reduce the crude fat content of Selig leaves flour when fermented for 5 days.

Decreased crude fat content in fermented Seligi leaves flour can be caused by the breakdown of triglyceride complex bonds into simpler bonds, among others in the form of fatty acids and glycerol needed in the metabolic process. According to Poedjiadi and Supriyanti [14], bacteria can break ester bonds in fat becomes fatty acids and glycerol. Some of the fatty acids formed will evaporate so the crude fat content decreases. Amrullah et al. [15] state that the crude fat content of feed ingredients consists of glycerol esters, fatty acids and fat-soluble vitamins that make it volatile. According to Kusumaningrum et al. [16], crude fat reduction in fermented feed ingredients can also be caused by the substrate used to contain glucose so that it can spur the growth of biomass which results in the production of more lipase enzymes to remodel the crude fat. The improvement of crude fat content can be caused by the occurrence of the process of degradation of organic material used by bacteria to form fat so that the levels of crude fat have increased [17].

4. Conclusions
Based on the results obtained in the study of fermented Seligi leaf flour using probiotics, it can be concluded that the addition of probiotics with different duration and doses affects the content of crude protein and crude fat in Seligi leaves and there is no interaction between the fermentation duration and probiotic dose on the content of crude protein and crude fat in the Seligi leaves (P. buxifolius).

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