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Blood Health Indicators Changes Induced by Replacing Fish meal with Lentil seed meal in the Common Carp (*Cyprinus carpio* L.)

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ABSTRACT

According to the research, substituting a fish meal with a plant protein source in a fish diet presents several difficulties. As a result, the goal of this study was to evaluate the effects of substituting lentil seed meal meal for fish meal in the diet of *Cyprinus carpio* using a variety of blood health indices. Five different diets were created by replacing FM with 0 (control), 5, 10, 15, and 20% lentil seed meal meal. A total of 150 common carp with an initial weight of 103.8±0.5g are randomly distributed into 15 tanks for 70 days, at a rate of 3% live weight per day and two times each day. Blood samples were obtained from five fish in each tank at the end of the feeding trial to analyze hematological and biochemical parameters. Hematological and differential leukocyte counts showed no significant changes ($p<0.05$). Also, dietary treatments had no effect on serum glucose and total proteins (Albumin and Globulin). However, serum enzyme activity (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)), triglycerides, and total cholesterol were increased significantly ($p<0.05$) as the amount of lentil seed meal meal in the diet grew. According to the findings of this study, the maximum amounts of lentil seed meal replacement in *Cyprinus carpio* diets might be between 15 and 20% of total protein sources without causing adverse effects on hematological and certain plasma biochemical markers.

Keywords: *Biochemical, Common Carp, Hematology, Plant Protein*

ABSTRAK

Menurut hasil penelitian, penggantian tepung ikan dengan sumber protein nabati dalam makanan ikan mengalami beberapa kesulitan. Oleh karena itu, tujuan dari penelitian ini adalah untuk mengevaluasi efek dari penggunaan tepung biji lentil untuk menggantikan tepung ikan (FM) dalam diet *Cyprinus carpio* menggunakan berbagai indeks kesehatan darah. Lima diet berbeda dibuat dengan mengganti FM dengan 0 (kontrol), 5, 10, 15, dan 20% tepung biji lentil. Sebanyak 150 ikan mas dengan berat awal 103,8±0,5g didistribusikan secara acak ke dalam 15 tangki selama 70 hari, dengan bobot pakan 3% bobot hidup per hari dan dua kali sehari. Sampel darah diperoleh dari lima ikan di setiap tangki pada akhir percobaan pemberian pakan untuk menganalisis parameter hematologi dan biokimia. Data hematologi dan differensiasi leukosit tidak menunjukkan perubahan yang signifikan ($p<0,05$). Juga, perlakuan diet tidak berpengaruh pada glukosa serum dan protein total (Albumin dan Globulin). Namun, aktivitas enzim serum (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)), trigliserida, dan kolesterol total meningkat secara signifikan ($p<0,05$) seiring dengan bertambahnya jumlah tepung biji lentil dalam makanan. Menurut temuan penelitian ini, jumlah maksimum penggantian tepung biji lentil dalam diet *Cyprinus carpio* sebaiknya berkisar antara 15 dan 20% dari total sumber protein tanpa menyebabkan efek buruk pada penanda biokimia darah dan plasma tertentu.

Kata kunci: Biokimia, Ikan Mas, Hematologi, Protein Nabati

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1. Introduction

The common carp (*Cyprinus carpio* L.) is an omnivorous fish that is widely cultivated across the world due to its rapid growth, disease resistance, and tolerance to a wide range of water quality and temperature. This species' omnivorous eating habit made it easy to adapt to plant-based diets, which can reduce the cost of production (Jain, 2002; Pillay and Kutty, 2005).

Fish meal (FM) is a high-protein source for fish. Because the catch fisheries are leveling off, many nutritionists are discovering that the existence of fish meal (FM) cannot be guaranteed any longer, therefore the idea of increasing the use of vegetable protein sources in the diet has been studied (FAO, 2011). Several possible fish meal substitutes, such as plant proteins, have been researched and tried for use in aquaculture during the last few decades. When choosing fish meal substitutes, it's crucial to think about their health impacts. Hematology, innate immunological responses, plasma/serum protein, and liver enzymes (e.g., ALT, AST, and ALP) can all be used to assess this condition (Sheikhlar *et al*., 2018).

Blood parameters are important indicators of a fish's physiological stress response and general health (Kader *et al*., 2012). Several variables, including fish species, life stage, environmental upbringing, and dietary regime, are predicted to influence hematological and biochemical parameters in fish (Osuigwe *et al*., 2005). Nutritional deficiency and imbalance are important factors that can induce changes in the blood parameters of fish (Hisano *et al*., 2016).

Grain legumes are a well-known dietary protein source in many parts of the world. Lentil seed meals are a rich source of plant-based proteins, with a crude protein concentration of about 26% on average, and can be used to replace animal and soybean proteins in diets (Khazaei *et al*., 2019). Some researchers concluded that lentils may be used to substitute fish meal in the diets of *Oncorhynchus mykiss* and *Cyprinus carpio* if the fish's overall performance was not affected when they were fed plant-based meal (Yürüten Özdemir and Yıldız, 2019; Abdulrahman and Abdulla, 2020; Abdulrahman *et al*., 2021).

In light of the growing usage of plants as an important source of protein in many cultures, it's time to examine their impact on several hematological and biochemical parameters to determine the amount of plant protein in the diet that might harm the body's systems. The purpose of this study was to evaluate how partial replacement of fish meal with lentil seed meal meal in the diet of common carp affected various blood health indicators.

2. Material and Methods

2.1. Fish and experimental procedure

This research is a continuation of one that was previously published by Abdulrahman *et al.* (2021). In this study, a total of (310) common carp fish with an initial weight of 105±5g were bought from a local fish farmer (Sulaimani/ Iraq). The fish were taken to the University of Sulaimani's College of Veterinary Medicine's laboratory. When the fish arrived in the lab, they were sterilized for 8-10 minutes with NaCl 3%, for the aim of adaption to the experimental environment, the fish were given a commercial diet (28% crude protein) before the experiment for four weeks duration.

The fish were kept in a rectangular research tank (fifteen plastic tanks filled with 100 liters of water) that was supplied with dechlorinated tap water at a daily exchange rate of 1/2 tank volume and had appropriate continuous aeration provided by stone bubbles connected to the central air compressor. The tanks were kept in good condition by using natural photoperiod settings and ambient light levels from daylight entering the research lab windows. The temperature of the rearing water ranged from 25 to 28°C during the experiment, dissolved oxygen was 6-8 $mg \cdot L^{-1}$, and the pH of the water was between 7.5 and 8. All of the prior quality of the water is within acceptable levels for fish growth (Boyd and Tucker, 1998).

This experiment involved three replications of four treatments and a control treatment, with 150 fish distributed randomly into 15 tanks, each weighing 103.8±0.5g (mean ± S.D), only normal and visually healthy fish were chosen, and the trial lasted 70 days. The tanks were monitored for mortalities regularly, and any dead fish were removed and weighed. Regular cleaning via siphoning was used to remove leftover feed and excrement from the system.

2.2. Diet formulation

The diets were made in the lab with local fodder materials (soybean meal, yellow corn, barley, wheat bran, and lentil seed meal meal), which were crushed and mixed until homogeneous, then it was split into five parts for treatment diets, with equal weights of a lentil seed meal (LS) replacing equal weights of the fish meal at 0% (control), 5% (LS5), 10% (LS10), 15% (LS15), and 20% (LS20),

Table 1. Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	Fishmeal replacement levels (%) with lentil seed meal (LS)				
	control (0%)	**LS5 (5%)**	**LS10 (10%)**	**LS15 (15%)**	**LS20 (20%)**
Soybean meal (48% crude protein)	35	35	35	35	35
Yellow Corn	13	13	13	13	13
Barley	15	15	15	15	15
Wheat	20	20	20	20	20
Fish meal (65.4% crude protein)	15	14.25	13.5	12.75	12
Lentil seed meal [1] (25% crude protein)	0	0.75	1.5	2.25	3
Vitamin + mineral mixture [2]	2	2	2	2	2
Total	100	100	100	100	100
Proximate analysis (% of dry matter basis) [3]					
Crude protein (%)	28.46	28.31	27.89	27.39	26.94
Crude fat (%)	3.2	2.1	2	2	1.94
Crude Fiber %	4.7	4.8	5	5	5.1
Dry Matter	87.4	87.8	90.7	92.1	92.7

Note: [1] According to the USDA National Nutrient Database; [2] Vitamin and mineral mixture according to Abdel-Tawwab et al. (2021); [3] Determined according to the standard methods of AOAC (2005).

respectively, as indicated in Table 1. The nutritional components were combined with water to form a paste, and then the pellet size of 3.0 mm was created using meat grinder mesh. For ten weeks, fish were hand-fed to satiation two times each day (10:00 a.m. and 03:00 p.m.) at a rate of 3% live-weight per day. Every two weeks, the fish in each tank were weighed, and the feeding quantities were changed based on the new weights.

2.3.Blood sample collection and preparation

Five fish per replicate were selected as samples at the end of the feeding trial and anaesthetized with clove powder (200)mg/L (Hassan *et al*., 2021), and blood was collected by cutting the caudal peduncle and placed in the two classes of Eppendorf tubes. For assessing CBC count parameters (Complete Blood Count), one set contains sodium heparinase (20 $U.L^{-1}$). The second set received no anticoagulant and was allowed to clot at 4°C before being centrifuged at 5000 g for 20 minutes at the temperature of the room to collect serum for biochemical tests.

2.4. Hematological indices

All hematological tests were performed with a Mindray BC-2800 China-made hematology analyzer, which is a completely automated hematology analyzer with 19 parameters for the Complete Blood Count (CBC) test (Abdulrahman *et al*., 2019). The parameters measured were as follows: hematocrit (HCT), erythrocyte counts (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), hemoglobin (HB), and differential leukocyte count (WBC) (granulocytes%, lymphocytes%, and monocytes%.)

2.5. Serum biochemical indices

Serum biochemical analyses including glucose, total protein (TP), globulin, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), and very-low-density lipoprotein (VLDL) were measured using an automated chemical analyzer and commercial kits from Spinreact, S.A. (Gerona, Spain) follows the method described by Abdel-Tawwab *et al*. (2020).

2.6. Statistical analysis

A randomized design was used to assign all diets. The mean±standard deviations (SD) are used to show the data. When significant differences ($p<0.05$) were discovered in all of the data, the Duncan Multiple Range test was performed to determine the group means. SPSS V.20 was used to conduct the statistical analysis.

3. Results and Discussion

Table 2 shows the results of several hematological markers after 10 weeks of feeding with diets containing variable quantities of lentil seed meal meal. According to the findings, no significant ($p<0.05$) differences

Table 2. Hematological parameters of common carp given the experimental diets for 70 days, each value is shown as Mean±SD.

Parameters	Levels of a lentil seed meal (LS%) within experimental diets				
	Control	LS5	LS10	LS15	LS20
RBC (10^{12} cells·L^{-1})	0.81±0.48	0.98±0.32	0.91±0.1	0.88±0.3	0.80±0.04
HB (g·L^{-1})	99.5±0.16	80.1±0.16	80.5±0.03	88±0.08	83±0.1
HCT (%)	16.6±0.47	19.8±0.17	19.5±0.06	18.9±0.34	15.5±0.01
MCV (fl)	212.3±0.01	205±0.16	224.5±0.04	213.9±0.05	208.1±0.03
MCH (pg)	122.8±0.62	81.7±0.17	88.5±0.13	100±0.37	103.8±0.14
MCHC (g.L^{-1})	599.3±0.61	404.5±0.02	412.8±0.09	465.6±0.41	535.5±0.11

***Note:** Different superscript letters indicate significant changes in the same row. If there are no letters, there are no treatments varied significantly (Duncan's test, p<0.05). hematocrit (HCT), erythrocyte counts (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).*

Table 3. Differential leukocyte count of common carp given the experimental diets for 70 days, each value is shown as Mean±SD.

Parameters	Levels of a lentil seed meal (LS%) within experimental diets				
	Control	LS5	LS10	LS15	LS20
WBC (10^9 cells·L^{-1})	219.6±0.01	189.7±0.08	191.1±0.07	198.6±0.07	189.8±0.09
Lymphocytes (%)	11.5±0.02	8.3±0.06	9.7±0.07	10.1±0.08	14.7±0.04
Monocytes (%)	33.6±0.07	36.4±0.05	35.1±0.02	36.8±0.05	38.1±0.04
Granulocytes (%)	54.9±0.05	55.3±0.1	55.2±0.3	53.1±0.05	47.2±0.2

***Note**: Different superscript letters indicate significant changes in the same row. If there are no letters, there are no treatments varied significantly (Duncan's test, p<0.05). Differential leukocyte count (WBC).*

were detected in the measured hematological parameters (RBC, HB, HCT, MCV, MCH, and MCHC) in the tested fish. Some parameters were greater in the different experimental groups than in the control groups, such as RBC and HCT in the LS5 group, while others were lower, such as HB, MCV, MCH, and MCHC in the examined group that ate the LS5 diet. None of this variation, however, was statistically significant ($p<0.05$).

The differential WBC count of the studied common carp is shown in Table 3. WBC was reduced in all tested groups as compared to the control group, even though the difference was not statistically significant ($p<0.05$). Furthermore, there were no significant changes in assessed leukocytes (granulocytes, lymphocytes, and monocytes) between the treatment and control fish groups.

The results demonstrated no significant differences in plasma glucose, total protein (TP), globulin, and albumin levels across the treatments after 10 weeks of feeding common carp with different levels of lentil seed meal. According to the findings, diets containing varying amounts of lentil seed meal induced a significant rise in serum enzyme levels such as alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase especially in treatment four (LS15) and treatment five (LS20), as shown in table 4. The highest ALT (68.9 IU.l^{-1}) and AST (576.9 IU.l^{-1}) were detected in the LS20, which contained a 20% replacement of fish meal with lentil seed meal. Treatment LS15 also detected the highest ALP (20.15 IU.l^{-1}).

The serum lipid profile of treated common carp is shown in Table 5. According to the findings, dietary lentil seed meal levels had a significant ($p<0.05$) influence on cholesterol, triglyceride, and VLDL levels with the fish fed the control diet having the lowest values of the aforementioned parameters in the majority of situations. However, the levels of HDL and LDL showed no significant variations.

In aquaculture, veterinary practice, and scientific study, hematological analyses are routinely used to assess fish health and welfare. cThe complete blood cell count (CBC) is measured in peripheral blood tests, which are frequently complemented by biochemical analysis (Fazio, 2019). In the present study, hematological parameters (HCT, RBC, MCH, MCHC, MCV, and HB) remained unaffected by lentil seed meal. These findings are consistent with those of Nasr *et al*. (2021) that substituting fish meal in the *Clarias gariepinus* diet with plant protein meal (soybean and sunflower meal) did not influence hematological variables. Rahmdel *et al*. (2018) showed that replacing up

Table 4. Serum constituents of common carp given the experimental diets for 70 days, each value is shown as Mean±SD.

Parameters	Levels of a lentil seed meal (LS%) within experimental diets				
	Control	LS5	LS10	LS15	LS20
Glucose (mg.dL^{-1})	72.5±0.19	77.4±0.33	79.2±0.5	73.8±0.05	75.6±0.02
TP (g.dL^{-1})	31.6±0.13	33.4±0.15	30.2±0.3	36.3±0.26	36.2±0.12
Albumin (g.dL^{-1})	1.5±0.04	1.8±0.1	2.1±0.15	2.2±0.17	2±0.13
Globulin (g/.dL^{-1})	30.1±0.13	31.6±0.18	28.1±0.3	34.1±0.27	34.2±0.12
ALT (IU.l^{-1})	33.7^{c} ±0.11	43.95^{b} ±0.14	47.45^{b} ±0.16	47.9^{b} ±0.19	68.9^{a} ±0.17
AST (IU.l^{-1})	281.05^{c} ±0.03	276.8^{c} ±0.05	417.3^{b} ±0.08	451.25^{b} ±0.04	576.9^{a} ±0.09
ALP (IU.l^{-1})	17.7^{b} ±0.03	17.6^{b} ±0.09	16.3^{b} ±0.08	20.15^{a} ±0.04	19.5^{a} ±0.07

***Note**: Different superscript letters indicate significant changes in the same row. If there are no letters, there are no treatments varied significantly (Duncan's test, p<0.05). Total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP).*

to 75% of fish meal for common carp fingerlings with sunflower meal is possible without negative impacts on hematological indices. Furthermore, Hosseini Shekarabi *et al*. (2020) recommended that fishmeal can be substituted with corn protein concentrate in rainbow trout diets without affecting hematological characteristics. In contrast to our findings, some other studies have indicated that replacing fish meal with herbal-based protein sources can result in a reduction in hematological parameters and, as a result, a decline in the health status of treated fish but not below normal levels (Ozovehe, 2013; Potki *et al*., 2018; Sheikhlar *et al*., 2018; Hernández *et al*., 2021). Physiological stress is created when dietary protein levels are low, which affects the liver, resulting in lower RBC and Hb concentrations (Hlophe Samkelisiwe and Moyo Ngonidzashe, 2014).

In vertebrates, the leukocyte count (WBC) is an essential metric in determining the immunological state. Differential leukocyte count is one of the most commonly used and readily analyzed indicators to learn about fish health from blood (Witeska *et al*., 2022). There was no difference in differential leukocyte count in *C. carpio* given the treatment diets, indicating that the inclusion of lentil seed meal as a partial substitute for FM (fish meal) should be validated. This finding is in line with previous findings by Sheikhlar *et al*. (2018), Kumar *et al*. (2010), and Soltanzadeh *et al*. (2015) who stated that there is no significant relationship when the dietary fish meal is replaced with plant-based protein meal (fenugreek seed, detoxified kernel meal, and faba bean) respectively, and differential leukocyte counts of blood in the treated fish groups. Aside from these findings in the literature, many other studies have found that replacing FM with plant-based protein meals can cause significant changes in WBC count due to the presence of antinutritional substances in many herbal proteins, which can have negative effects on the fish's immune cells and defense system. (Jahanbakhshi *et al*., 2012; Jalili *et al*., 2013; Potki *et al*., 2018).

Hematological and biochemical indices provide extensive information about fish oxygen transport capacity, immune potential, level of stress, disease, intoxication, nutritional status, etc. (Witeska *et al*., 2022). In the current study,

Table 5. Serum lipid profile of common carp given the experimental diets for 70 days, each value is shown as Mean±SD.

Parameters	Levels of a lentil seed meal (LS%) within experimental diets				
	Control	LS5	LS10	LS15	LS20
TG (mmol.L^{-1})	173.4^{b} ±0.31	181.1^{b} ±0.29	241.3^{a} ±0.38	244.9^{a} ±0.13	236.1^{a} ±0.29
CHOL (mmol.L^{-1})	88.1^{b} ±0.02	88.9^{b} ±0.1	101.3^{a} ±0.2	104.1^{a} ±0.12	102.6^{a} ±0.08
HDL (mmol.L^{-1})	46.5±0.13	46.4±0.11	45.9±0.17	48.1±0.14	48.4±0.19
LDL (mmol.L^{-1})	6.9±0.34	6.3±0.42	6.1±0.26	7.1±0.51	7±0.46
VLDL (mmo.L^{-1})	34.7^{b} ±0.31	36.2^{b} ±0.3	49.3^{a} ±0.39	48.9^{a} ±0.13	47.2^{a} ±0.3

***Note**: Different superscript letters indicate significant changes in the same row. If there are no letters, there are no treatments varied significantly (Duncan's test, p<0.05). Triglycerides (TG), total cholesterol (CHOL), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and very-low-density lipoprotein (VLDL).*

LSM levels did not influence glucose levels in all treatments. This finding is in line with previous publications (Slawski *et al*., 2011; Hlophe Samkelisiwe and Moyo Ngonidzashe, 2014; Potki *et al.*, 2018). In general, glucose level is a stress indicator to demonstrate the fish's health status (Falahatkar and Poursaeid, 2014). The lack of alterations in glucose in this investigation revealed that the treated common carp were in good health.

The total protein content in the blood is an essential metric for the purpose of keeping a check on the nutritional status and health of fish. Albumin and globulin are two main blood proteins that have a role in the immune response. As a result, any change in their levels suggests a lack of nutrients, immunostimulating effects, and a problem with protein metabolism in the liver (Silverman *et al*.,1986; Martinez, 1976). Blood total protein levels, including albumin and globulin, were not statistically significant across the dietary groups in this study. These findings are in line with prior research on plant protein replacement in juvenile Gibel carp (Zhang *et al*., 2020) and Red sea bream (Linn *et al*., 2014). However, fish meal substitution with peanut meal in the diet of Rainbow trout (Acar and Türker, 2018) and Moringa leaves meal in the diet of *Clarias gariepinus* (Hlophe Samkelisiwe and Moyo Ngonidzashe, 2014) resulted in a reduced trend in total blood protein levels.

Serum enzyme levels can be utilized as health markers in a variety of animal species, including fish. They are required for signal transduction and cell control, and are frequently mediated by kinases and phosphatases. Alkaline phosphatase, alanine aminotransferase, aspartate, and aminotransferase activity are effective markers of liver and kidney damage (Akanji *et. al*., 1993).

The activity of serum enzymes (aspartate aminotransferase AST and alanine aminotransferase ALT) increased significantly ($p<0.05$) as the amount of lentil seed meal meal in the diet grew, and so did the levels of alkaline phosphatase ALP. Previously, high replacement levels of FM with *Moringa oleifera* leaf meal in Juveniles African catfish (Ozovehe, 2013) and corn protein concentrate in rainbow trout (Hosseini Shekarabi *et al*., 2020) were observed to enhance plasma activities of ALT, AST, and ALP which in line with our results. While several studies saw a decrease in blood enzyme levels when they replaced FM with plant protein meals (Soltanzadeh *et al*., 2015; Zhang *et al*., 2020). The effects of FM replacement with LSM on ALT, AST, and ALP activities in different treatments suggest that replacement levels of FM with LSM may be high enough to cause hepatic cellular damage and leakage into the bloodstream. These outcomes might be attributable to an increase in the amount of anti-nutritional and toxic components found in plant proteins (Linn *et al*., 2014).

Plant proteins have been seen to influence lipid metabolism regulation in fish (Mesina *et al*., 2013). Studies on common carp found that using corn gluten meal instead of FM in diets resulted in higher serum lipid profile levels, similar to our findings (Potki *et al*., 2018). Furthermore, triglyceride levels in the blood in juvenile spotted rose snapper fed corn gluten protein diets were greater than those fed fish meal diets (Hernández *et al*., 2021). However , some researchers saw a decrease in the serum lipid profile levels of treated fish when plant protein sources were used instead of FM in diets (Rahmdel *et al*., 2018; Wang *et al*., 2020). Others, on the other hand, found that plant protein in the diet did not influence blood total lipid profile levels (Slawski *et al*., 2011; Sheikhlar *et al*., 2018). Triglycerides and cholesterol are produced primarily in the liver and are used to measure the physiological changes that occur when fish are fed for a short period (Bucolo and David, 1973). Essential amino acid deficiency can cause liver malfunction, increased lipogenesis, and impaired fatty acid beta-oxidation, resulting in significant increases in plasma lipid levels (Matter *et al.,* 2004).

4. Conclusion

The findings of this study demonstrated that it is feasible to substitute 20% of fish meal with plant-based protein (lentil seed meal meal) in *Cyprinus carpio* without causing significant changes in hematological and biochemical markers. Other plasma biochemical markers, such as serum enzyme activity and lipid profiles, are, however, disturbed. As a result, additional study is needed to assess common carp health when plant protein such as lentil seed meal is used instead of fish meal.

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