Biochemical Evaluation of Millet-Based Fish Feed and its Effect on *Danio rerio* (Zebrafish) on its Growth Parameters

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Abstract: ***A study was carried out to determine the effect of millet-based fish feed on the growth parameters of Danio rerio (Zebrafish). Fish were brought to the lab and acclimatized before the experimental trails. Then they were randomly divided into two groups, control and experimental. The Control group was fed with standard market bought dry fish feed and the experimental group was fed with lab made millet-based fish feed. The lab made fish feed was made using a known millet, Sorghum sp. which was germinated and then used to make the fish feed. Both fish feeds were tested for their biochemical components like proteins, carbohydrates and lipids. The growth parameters like length and weight were monitored periodically. It was found that the amount of protein was significantly higher in lab made fish feed (7%) than market bought fish feed (5.9%). The growth parameters varied significantly in both the feeds with higher weight and length gain in lab diet fed fishes (1.29g, 10.7mm) than market diet fed fishes (0.36g, 4mm). Thus, millet-based lab fish feed showed a significant positive effect on the growth parameters of zebrafishes with an increase in net weight and length gain of the fish. This study thus paves way towards the utilization of millet-derived feeds that increase the quality of fish. This can be applied in various fields like aquaculture, fishery etc., which can increase productivity using sustainable methods.***

Keywords: Millets, Fish-feed, Zebrafish, Animal Nutrition

1. Introduction

The taxing demand of maize and rice has led to search for alternative sources that are nutritionally sufficient and economically beneficial to society [12]. Millets are alternative grains which are widely grown in Asian and African countries. These grains are easily grown in conditions like droughts which are beneficial for various industries (food & feed) for raw material availability. Given the fact of current underuse of millets, it has led to research more about the nutritional profile of these grains and raise awareness amongst society about its health benefits. Some review articles in millets have mentioned the urge to route for millet and sorghum instead of maize and other major crops in recent years [11]. It is derived from the fact that these grains are ecologically well-matched with semi-arid areas because of their ability to tolerate drought. These crops are considered tough crops in terms of growth requirements as they withstand harsh climatic factors such as unpredictable climate and nutrient-depleted soils" [22].

Millets are significant cereals referred to as superfoods (approved by UN 2023; international year of millets) as they are packed with proteins, fibers, and vitamins are considered as a major source of macronutrients essential for growth and development of fishes as well as humans. According to researchers, a diverse variety of millets are proven to be a major source of nutrients, minerals, and vitamins; they are gluten free, with high phenolic properties (phenolic acids, flavonoids, and tannins) having higher antioxidants activity than conventional grains.

Millets significantly contribute to human food and fish feeds owing to their composition having phosphorus, manganese, magnesium, calcium, iron, Vitamin B2, B3, B5, B6, C and K etc. Millets have 65-75% carbohydrates, 9-12% proteins, 3-7% fat, and 2-8% crude fiber [12]. As a result, the use of plant-based proteins (millets) in fish feeds came into light. This will not only increase commercial value and market for millets but also will alleviate some pressure of creating rice and maize production. The proficient fish feed is the heart and soul of aquaculture systems for maximum production of fishes in the shortest amount of time possible. These feeds play an important role that ensures optimal growth for different fish species reared under different conditions [25]. The use of plant-based proteins is utilized to maintain healthy and fast growth which is much needed to fulfil increasing demands of fish feed for fish aquaculture. As feed is important for an economically productive aquaculture system, we need to make feed that is cost effective and nutrient rich for healthy growth of fishes [2]. Thus, using millets can be an economically viable option for producing nutrient rich fish food.

Fish feed can be categorized into traditional, commercially produced and fusion of both feeds. The artificial or commercially produced feeds are mostly of complete diet consisting of all the indigenous nutrients necessary to reach optimal growth promptly. The composition of this feed is approximately: protein 18-20%, carbohydrates 15-20%, lipids 10-25%, ash <8.5 %, phosphorus <1.5 %, water <10 %, and trace amounts of vitamins and minerals [10]. Meanwhile, traditional fish feed contains fish meals and fish oil as the major ingredients. Carbohydrates can be used in limited amounts for energy along with small quantities of vitamin and mineral mixtures and other additives. Fish oil is a naturally depleting source which is expected to reach the world maximum level of production of about 1.1 million tons. This is just another reason which hints towards use of alternative plant-based proteins and lipids for energy and developmental purposes [20]. The plant-based feeds are eco-friendly, sustainable and cost effective as it utilizes millets (prevents their underuse), also replaces fish-based products (hard to digest for some omnivorous or other fish) with ingredients like soybean powder and spirulina which are well balanced diets and have content of protein, amino acids, taurine, lipids, and pigments. There is also the presence of carotenoids which is essential for reproduction and natural pigmentation of animals.

In the present study, zebrafish (wild type) was chosen as a model organism. These fish were acclimatized in tanks under lab conditions and were fed with two types of diet (feed). The control diet consisted of Market bought feed and the experimental diet consisted of Millet based lab feed. Regular two-time feeding was done, and tanks were checked periodically for parameters like pH and temperature of water. The comparative analysis of biochemical compositions for protein, carbohydrate and lipid content was done of both the diets and then its effects on the growth parameter was measured.

2. Materials and method

In the current study zebrafish were fed with lab made feed made from millets and regular marketed fish feed.

2.1 Germination of millet (*Sorghum sp*.)

Germination was done in the simple method by rinsing the millets thoroughly to remove unwanted particles and allowing them to be kept in a moist environment by pre-soaking the millets overnight. The millets were laid on a clean moist cotton cloth. The germinates appeared within 3-5 days. Research has shown that anti-nutrients are found in high amounts in a variety of millets, which makes the micronutrients less bio accessible [9]. This problem can be overcome by processes such as germination. According to Abioye *et al*, germination increases the nutritional qualities of millets [1]. Hence. germination was an added step in the process of making fish feed.

2.2 Diet formulation

Experimental (Lab feed: millet-based): The germinated millets were grounded to get consistency forming the millet meal. Vegetables, in particular spinach, carrot and green peas were chosen to provide requisite nutrition such as vitamins, carotenoids, and antioxidant properties. The external peel of peas was discarded since they are not easily digestible for the fish. Carrots and green peas in equal parts were boiled, while the spinach (1 part) was blanched to form the formulated meal. Mashed fish (Bombay duck) without bones were mixed along with the equal parts of spirulina and soybean powder. The formulated meal was blended along with the millet meal (30%) to form fish feed. The mixture made by blending the components was made into a smooth consistency and they were evenly laid approximately 0.5m in thickness. They were shade dried and crushed to obtain flakes of size edible to be fed to the fishes. Dried feed was stored in an airtight container until further use.

Control (Market feed): A standard commercially available dried fish feed 'Aini Fish Food' was taken as control feed.

2.3 Experimental protocol and tank set up

Danio rerio were used for feeding trials. Forty fish were used in this study. The fish were starved for 72 hours for acclimatization before the feeding trails. 10 fish were randomly selected and placed in transparent aquaria. The initial lengths and weights of the selected fishes were measured and recorded for replicates. The fish were then randomly divided into two groups, control and experimental with not more than 10 fish per tank. The experiments were conducted in the tanks with water of about 80%. They were fed twice with the respective diets (experimental and control), twice daily at 10% body weight between (7.00 a.m. to 6.00 p.m.) for 21 days. The tanks were provided with continuous aeration. The parameters like pH, temperature and ammonia concentration were checked daily. Temperature was maintained between the range of 27°C to 29°C. The pH range was between 6.5 to 7.5. Ammonia concentration in the tank was well monitored for the appropriate development of fishes. The length and weight of these fish was recorded in a periodical manner [3]. Biochemical estimation of both the feeds was also performed. The entire experiment was conducted in the Set of three to get statistical data.

2.4 Biometric data

Fish's length and weight were estimated carefully to understand the growth pattern. Fish were measured in terms of weight gain and increase in length. Total length was measured to the nearest 0.1 mm using a 30 cm ruler as the distance from the tip of the anterior most part of the body to the tip of the caudal fin. Analytical balances with precision of 0.01 g were used to record body wet weight [17]. At the conclusion of the experiment, both weight (g) and length (mm) were determined to calculate condition factor indices $K = [\text{weight}\times100]/\text{length}^3$ [14].

2.5 Biochemical analysis of fish feeds

A comparative analysis of the content of protein, carbohydrate and lipids were done for the control and experimental fish feeds.

2.5.1 Total protein content

The total protein estimation was done using the Folin Lowry method [19]. The two feeds were subjected to hydrolysis by addition of a phosphate buffer (pH 7) of about 10 ml each to the control and experimental feeds. The samples along with blank containing distilled water and standard which is BSA were treated with reagents according to standard protocol. The tubes were then incubated for 10 minutes at room temperature. Folin's reagent was freshly prepared and added

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to the samples and the absorbance was measured colorimetrically at 660 nm [21].

2.5.2 Total carbohydrate content

The total carbohydrate content was estimated by the Anthrone method [13]. The control and experimental samples were hydrolyzed with 3 ml of 2.5N HCl. Later it was neutralized using sodium bicarbonate until no effervescence appeared. The samples were diluted up to 100 ml volume and from which 1 ml was taken. The samples along with blank containing distilled water, standard which was glucose standard each of 1 ml volume were treated with freshly prepared anthrone reagent. The absorbance was measured colorimetrically at 660 nm.

2.5.3 Total Lipid content

Lipid content was estimated by Bligh and Dyer method [7]. 0.1g of sample of both control and experimental was treated with 1:2 Chloroform: Methanol reaction mixture. The samples were homogenized for 2 minutes and blended in a vortex mixture to get a high degree precise homogenized blend. In addition, 0.1 ml of chloroform was added and blended in a vortex mixture for 30 seconds. Further 0.1 ml of distilled water was added to the homogenate which was blended for 30 seconds more. The mixture was filtered using the Whatman No.1 filter paper under slight suction. The samples were collected in a calibrated cylinder which were allowed to stand for some time till phase separation was seen. Once complete separation was observed the chloroform layer was recorded along with the total volume of aliquot. The upper methanolic layer containing hydrophilic compounds was removed along with a part of impurities of the chloroform layer. The samples were allowed to evaporate the chloroform from the samples resulting in the lipid being left out that was later weighed. The lipid content was calculated using the formulated method.

2.6 Statistical analysis

Data was presented as mean ± S.D and analyzed by Student t-test. P values of less than 0.05, 0.01 and 0.001 were considered statistically significant.

3. Result and Discussions

The analysis was based on a comparative study of millet-based fish feed (experimental feed) and market feed (control feed) to recognize the nutritional profile and comprehend which feed is preferable for obtaining optimum growth. The results obtained from the evaluation of the proximate composition comprise proteins, carbohydrates, and lipids of the samples.

3.1 Growth parameters

The growth performance of the fish was analyzed by measuring the weight and length gain by the fishes in experimental and control groups.

3.1.1 Weight analysis

Results of growth response and fish feeding data for *Danio* zebrafish are shown in Figure 1. There was a marked increase in weight gain observed in the experimental set up, which was fed with a lab-based diet. The highest average weight gain was observed in fishes fed with lab feed with 1.29 g weight gain and that of market feed was 0.36g. The significant difference in average weight gain suggests that fish fed with lab-based millet rich diets utilized their feeds successfully. The millet-based fish feed (lab- based feed) positively impacted the development of Zebrafishes, which was reflected in the growth ratio.

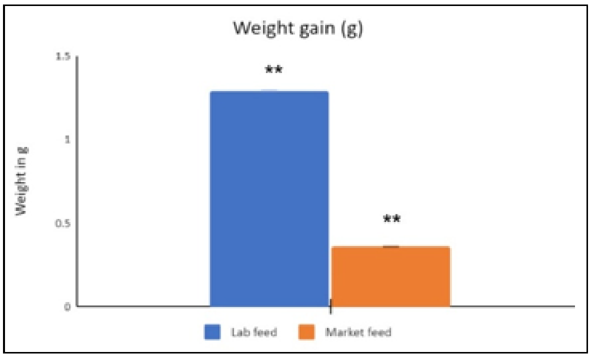

Figure 1: Average gain in weight (g) (Comparative analysis of average gain in fish weight (g) after-lab feed and market feed) Student t-test, *p≤0.05 and **p≤0.01.

3.2.3 Length analysis

Measurements of weight and length give us an idea about the growth parameters of the fish and allows us to understand whether a particular diet is well suited to the fish. Thus, length becomes an important parameter in fish feed studies. The average length gain at the end of the experiment in the lab feed (experimental) and market feed (control) group of fish is shown in Figure 2. It was observed that the average length gain in lab diet fed fish (experimental group) was 10.7 mm, which was much higher than the market diet fed fishes (control group) which was 4 mm.

Thus, this indicated that the lab made feed impacts the growth positively and leads to more gain in weight and length of the fish as compared to the market feed.

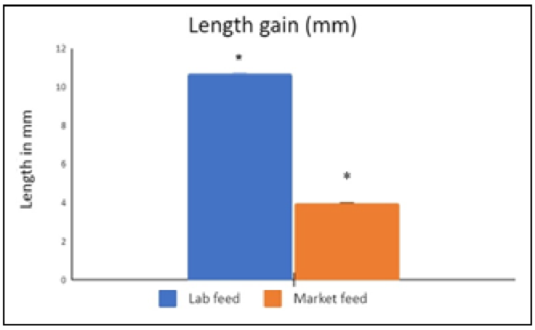

Figure 2: Average gain in length (mm) (Comparative analysis of average gain in fish length (mm) after lab feed and market feed) Student t-test, *p≤0.05 and **p≤0.01.

3.3 Condition factor indices

The condition factor (K) of a fish reflects upon the physical and biological circumstances and interaction among feeding conditions, parasitic infections and physiological factors [15]. It also indicates the changes in food reserves. It is therefore an indicator of the general fish condition. It is vital

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to know the condition factor indices of a culture management system as it provides us with information of the specific condition under which organisms are developing [4] [24].

The values of condition factor 'K' recorded in the present study are 1.21 for lab diet fed fishes and 0.94 for market diet fed fishes. Condition factors greater than one show the wellbeing of fish fed with experimental diet. The values of 'K' in lab diet fed fishes were higher than market diet fed fishes, suggesting that fish fed with a diet containing experimental diets were utilizing the feed better than the fish fed with market diet. The results are in conformity with the study of Siccardi, A.J., *et. al.*, who recorded the K value of *Danio rerio* to be a range of 0.91 to 1.43 [23].

3.2 Biochemical estimation of feeds

3.2.1 Protein estimation

Protein is the richest component in fish feed. Indispensable proteins act as a major component for the formation of tissues, organs, and structures in fishes undergoing embryonic and larval development. The main components of protein in lab feed were sorghum (millet), soybean and spirulina. Sorghum, a versatile and sturdy cereal grain, has an excellent nutritional profile, with protein being particularly significant. Sorghum plays a major role in achieving daily protein demands. Soybean meals have been greatly researched as a dietary ingredient to replace fish meals in the diets of various fish species, which is due to their high protein content, relatively balanced amino acid profiles, cost-effectiveness, and consistent availability [8].

The average protein content estimated using the Folin Lowry method was found to be 7% in lab feed and 5.9% in market feed (Fig. 3). Thus, an increased protein content in the lab feed is observed through the analysis compared to the market feed. This high protein content in lab feed may have attributed to the increased weight and length gain in lab diet (experimental group) fed fish.

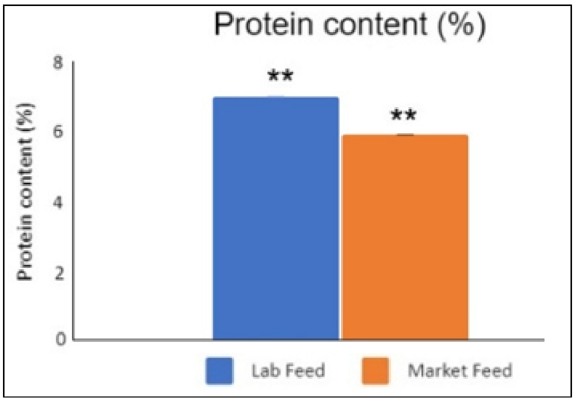

Figure. 3: Total Protein content (Comparative analysis of total protein content in lab feed and market feed) Student t-test, *p≤0.05 and **p≤0.01.

The protein content having the essential amino acids provides for the enhanced growth of fishes. Upon research on the nutritional profile of various grains, millets are said to contain ample amounts of nutrients such as proteins, carbohydrates, lipids, essential amino acids, micronutrients, fibers, and vitamins that satisfy the demands of satisfactory feed for aquaculture. The latest variety of sorghum with less tannins and more proteins improved the digestible energy of sorghum in diets of fishes resulting in complete utilization of feed ingredients. In a particular study an increased maturation rate of juvenile fishes was possible due to sorghum in particular which was valued for facilitating higher growth rate due to the presence of proteins and energy resources in abundant amounts [6]. Similarly in studies done by Liu T., *et. al.*, and Lee S.M., *et al.*, fish were fed with different levels of protein diets. It was found that appropriate protein levels could increase growth performance and feed utilization in the fishes [16] [18]. In the present study the high amount of protein content in millet-based Lab feed (experimental group) could have resulted in an increased weigh and length gain (Fig. 1 and 2) in the zebrafish.

3.2.2 Carbohydrate estimation

Carbohydrates are the main ingredient in fish feed because they are an essential source of energy and support number of physiological processes that are essential to fish growth and development. These macronutrients are essential for maintaining the overall fish vitality and for supplying the fuel required for metabolic functions, especially during the larval and embryonic phases of development.

The total carbohydrate content in the feed was estimated by using the Anthrone reagent method. Following the protocol, the results were found to be around 16% lab (Experimental) feed and 22% in market feed (Fig. 4). Millet is low in simple carbohydrates and high in complex carbohydrates. Commercial feed mostly contains nutrients above what the fish need. Due to this commercial feeds mostly show higher carbohydrate contents than millet-based fish feed. It was observed from previous studies that consumption of proteins and dietary energy was poorer in fish fed with elevated carbohydrate levels indicating that lower levels of carbohydrates provide better utilization of proteins and higher energy levels as seen in the lab-fed fish [5]. In fish feeds primarily the standard range for carbohydrates is seen to be 15-20% as fishes can utilize up to 20% of dietary carbohydrates [10]. Thus, the carbohydrate content in lab made fish feed is good for healthy fish growth as compared to the market feed used in the experiment.

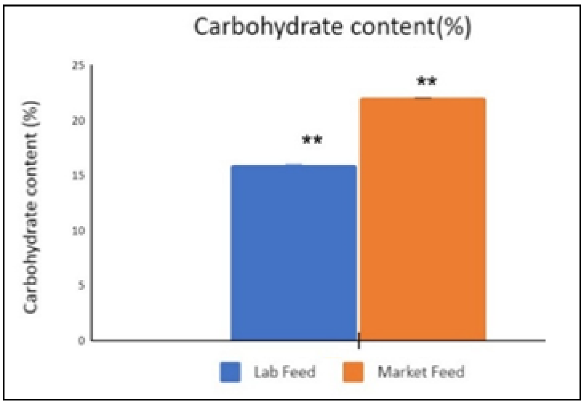

Figure 4: Total Carbohydrate content (Comparative analysis of total carbohydrate content in lab feed and market feed) Student t-test, *p≤0.05 and **p≤0.01.

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3.2.3 Lipid Composition:

The lipid content was evaluated through the Bligh and Dyer method [7]. The total lipid content was found to be 12.6% in market feed and 13% in lab feed (Fig. 5). Sorghum has a high nutritional value because it is high in unsaturated fats and proteins required in the dietary requirements of fish. When infused with shrimp (rich in omega-3 fatty acids), spirulina (rich in linolenic-essential fatty acids) provided an adequate amount of dietary fats for optimum growth for fish. Zebrafish are freshwater poikilothermic fishes with a high need for monounsaturated and polyunsaturated fats in their diet to provide energy for diverse activities and to survive in various temperatures. Thus, the high lipid content in the lab feed would prove to be of benefit for the overall fish growth and health.

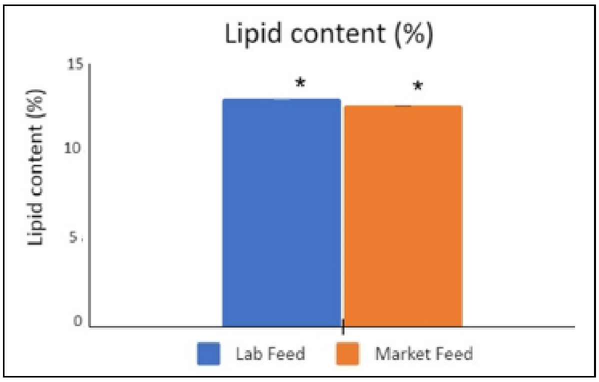

Figure 5: Total Lipid content (Comparative analysis of total lipid content in lab feed and market feed) Student t-test, $*p \leq 0.05$ and $**p \leq 0.01$.

In the present study we found that lab feed has more protein content and lipid content as compared to carbohydrates than market feeds.

4. Conclusions

From the overall discussion of the present experimental results, it has established that better growth of zebrafishes may be obtained using the feed with protein of millet-based origin. Millet based feeds are reinforced with natural nutrients which contribute to increase in growth and survival of fishes thus effectively making use of these underutilized grains. This study suggests that the prepared millet-based feed was effective in terms of faster growth and development as compared to commercially available feed. Therefore, future research should be focused on developing a commercial millet-based fish feed product and it can be experimented with other commercial fish species.

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