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EXPRESSION OF IMMUNE GENES AND STRESS ENZYME PROFILES OF RAINBOW TROUT *(ONCORHYNCHUS MYKISS)* FED *MORINGA OLEIFERA* LEAF MEAL (MLM)

Shyam Narayan Labh

Department of Zoology
Amrit Campus, Tribhuvan University, Kathmandu, Nepal
**Corresponding author:* shyam.labh@fulbrightmail.org

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Abstract: *Moringa oleifera* belongs to family Moringaceae, is one in every of the foremost useful trees within the world, with all the parts having nutritional and pharmacological properties. This research evaluated the impact of categorized nutritive insertion of *Moringa oleifera* leaf meal (MLM) on survival, growth, and antioxidant systems, with a few immune-related organic phenomena in rainbow trout. Fish diets formulated (M0, M10, M20, M30, and M40) to contain 0, 10, 20, 30 and 40% of MLM as protein source supplemented with fish meal respectively. The rainbow trout fishes (6.57± 0.56 g) were randomly distributed into 15 plastic tanks (n=15) and fed the same (twice daily) for 90 days. Growth parameters (WG%, SGR, and FCR), serum protein contents (TSP, TSA, TSG, and A: G), enzyme contents (SOD, CAT, GPx, AST, ALT) and organic phenomena, IL-6, IL-8, IL-10, and TNF-α genes were studied, compared to β-actin genes followed by blood morphology of trout. Fish fed MLM accompanied diet showed higher weight gain (349.31%) and SGR (1.35) and minimum FCR (1.33) values up to M20 (20%) group of fish fed MLM diet compared with control fish. Protein profile in blood and enzyme-like SOD, CAT, and GPx found higher while lower AST and ALT recorded altogether the treated groups compared to regulate. Similarly, MLM supplemented diets fed fish exhibited the expression of immune genes. Interleukin -6, Interleukin -8 and Interleukin -10 found significantly high ($P < 0.05$) compared to β-actin gene. Finally, this research showed that dietary supplementation of 20% MLM is perfect for effective growth performance, enhancing the antioxidant activities and answering some immune performance in rainbow trout (*Oncorhynchus mykiss*).

Keywords: Antioxidant, Growth performance, Immune response, *M. oleifera*, Rainbow trout.

INTRODUCTION

Aquaculture is the cultivation of organisms in the water and coastal areas under the culture process to solve the food protection problem in the world. It is undoubtedly the quickest growing sector (FAO, 2019). Over the times, aquaculture has not been without its share of problems even though its numerous advantages in producing a consistent protein supply to feed the planet people. Better nutrition and pollution free clean environment are necessary for sustainable development (Verma, 2019). One in all, aquaculture's result is the prevalence of antibiotics (Atal, 1982). Several other compounds (vitamins and hormones) analyzed in aquaculture procedures for numerous treatments. At the same time, they furnish positive effects, which cannot recommend expected to their enduring and other fringe sound effects. The repeated and sometimes unfettered uses of antibiotics have led to its mistreatment among the aqua farmers as a substitute for medicines, and environmentally secure (Citarasu, 2010). The substitute herbal foods need the qualities of expansion, stimulating capability, and stimulant to enhance the structure. The use of herbal products for fish disease management might be a promising alternative to antibiotics. Chemical drugs reported to stimulate appetite, growth performance, antioxidant, antibacterial, antiviral, and anti-parasitic and also act as immuno-stimulants in aquaculture due to the presence of phenols and flavonoids and saponins (Chakraborty and Hancz, 2011).

Moringa "The Queen of Green," commonly known by the 'drumstick tree', is taken into consideration as the full useful trees, as its every part used for food. The leaves, stems, and seeds are said to possess therapeutic uses and offer tribal family's revenue, foodstuff, and supplemented feed of animals. The tree may be about 10 meters high belonging to the Moringaceae family with a single genus having 13 known species. It may be ideal to fulfill the nutritious supplement of families (Makkar and Becker, 1997). In current years, *M. oleifera* has attracted the eye of researchers in agriculture to a greater extent. It is all because of MLM nutritional, antioxidant, and meditative characters.

The leaf of *Moringa oleifera* contains a large amount of protein, vitamin, mineral, and a carboxylic acid. It can provide protein nine folds extra than yogurt, calcium seventeen-fold more than milk, vitamin C seven-timesbetter than oranges, and ten times higher than carrots. Similarly, it provides iron twenty-five times more than spinach and potassium fifteen times higher than bananas (Gopalakrishnan *et al.,* 2016). The global market for herbal products has projected to have a yearly growth rate between five and fifteen percent. The overall macro herbal goodsare calculated as the 62 billion US dollar and expected to grow to five trillion US dollars by the year 2050 (Maggon, 2005). Medicinal herbs are as outdated as a civilization, and throughout history, they have used as popular folk medicine because of their wide-scale therapeutic assets (Bulfon *et al.,* 2013). With this background, current research performed on rainbow trout to estimate the MLM activities on growth execution, biochemical considerations, and some immune molecules, namely IL-6, IL-8, IL-10, and TNF-α in spleen tissues.

MATERIALS AND METHODS

1. Culture system:

This experiment was conducted at Aquaculture Research Unit under an outdoor flow-water system in which fifteen plastic tanks (30”x20”x 18”) were placed in five rows @ three tanks in a row. Juvenile rainbow trout procured from the local hatchery in trout village lies in Nuwakot of Kathmandu, Nepal, and acclimatized for fifteen days. During acclimatization, regular feed supplied to maintain the fish physiology. After that, two hundred and twenty-five fish (6.57± 0.56 g) were selected and distributed in all the fifteen tanks equally at 15 fish per tank. Fish fed treated and the control diet at the rate of 5% twice daily for 90 days.

2. Preparation of experimental diets:

The experimental diets (Table 1) prepared per the strategy explained by Labh *et al.* (2017) with slight modification. *Moringa* leaves (Figure 1) were obtained and soaked overnight in an exceeding tank to eliminate saponins and other water-soluble antinutritional factors. A wire mesh accustomed to drain excess water by placing the leaves on that, and then the soaked leaves were spread on plastic sheets to dry under shade to stop the loss of vitamins through photodynamic damage or oxidation. The dried leaves were threshed from stalks to cut back crude fiber content within the meal. The dried leaves were then subjected to grinding and converted them into a fine powder employing a hammer mill (Lab Mill, screen size 0.2 mm) and also the powder then stored in plastic bags at temperature. The excellent powdered MLM was used as a test ingredient to exchange costly fish meal and formulation of test diets with varying levels of replacement (Table 1). The feed ingredients were procured from an advertisement feed mill and analyzed for chemical composition following standard methods (Abu-Tarboush *et al.*, 1997) before the design of the treated diet. The ingredients were ground and sieved to need particle size before the innovative food (Table 1). The MLM utilized in the diet formulation by replacing fish meal at the speed of 0%, 10%, 20%, 30%, and 40%, and also, the feed given to 5 fish groups, which were stocked in water tanks having 70 L water capacity.

3. Examination procedure:

3.1. Growth performances:

In this study, fish growth assessed in terms of percentage Weight Gain (%), Specific Growth Ratio (SGR), and Food Conversion Ratio (FCR) calculated according to the following equations on 90th days of feeding followed by percentage survival on regular monitoring:

Figure 1: *Moringa* leaf meal (MLM).

WG% (g/fish) = [Average final weight — Average initial weight] / initial weight x 100

SGR = [ln (final weight in grams) — ln (initial weight in grams)] x 100 / t (in days).

FCR = Food intake / weight gain.

Survival % = Number of survived fish/initial number of fish x 100

(Note: All the fish weights in the given equations are calculated in gram units).

Table 1: Preparation of experimental diets and proximate analysis before feeding trials.

Ingredients	M0	M10	M20	M30	M40
Fish meal	25	22.5	20	17.5	15
MLM	0	2.5	5	7.5	10
Soya flour	20	20	20	20	20
Corn flour	12	12	12	12	12
Wheat flour	15	15	15	15	15
Rice bran	10	10	10	10	10
GOC	12	12	12	12	12
CMC	1	1	1	1	1
Veg. Oil	4	4	4	4	4
Vit & Mineral§	1	1	1	1	1
Total	**100**	**100**	**100**	**100**	**100**
Moisture	7.86±0.30	7.60±0.23	6.89±0.10	6.82±0.09	7.59±0.14
Ash	9.24±0.28	9.38±0.24	9.45±0.15	9.47±0.01	9.05±0.06
Crude protein	32.78±0.56	32.28±0.48	32.57±0.21	32.61±0.10	32.48±0.17
Ether extract	7.87±0.36	7.32±0.06	8.09±0.08	7.59±0.11	8.07±0.11
NFE#	39.06±0.25	39.11±0.20	38.07±0.20	37.74±0.17	36.17±0.18
CF	0.79±0.01	1.63±0.03	2.43±0.03	3.28±0.01	4.01±0.03
Digestible energy	370.95±4.05	368.65±0.86	375.09±0.26	372.76±0.86	373.79±0.40

Ingredients like fish meal, flour, cornflour, wheat flour, rice bran, GOC, CMC, veg oil, vitamins, and minerals were obtained from the local market of Kathmandu Valley.

§Composition of vitamin-mineral mix (EMIX PLUS) (quantity 2.5kg -1) Vitamin A 55,00,000 IU; Vitamin D3 11,00,000 IU; Vitamin B2 2,000 mg; Vitamin E 750 mg; Vitamin K 1,000 mg; Vitamin B6 1,000 mg; Vitamin B12 6 μg; Calcium Pantothenate 2,500 mg; Nicotinamide 10 g; Choline Chloride 150 g; Mn 27,000 mg; I 1,000 mg; Fe 7,500 mg; Zn 5,000 mg; Cu 2,000 mg; Co 450 mg; Ca 500 g; P 300g; L- lysine 10 g; DL-Methionine 10 g; Selenium 50 mgl-1; Selenium 50 mgl-1; Satwari 250 mgl-1; (Lactobacillus 120 million units and Yeast Culture 3000 crore units).

♯ Nitrogen Free Extract (NFE) = 100-(CP+EE+CF+Ash).

3.2. Biochemical profiles:
The spleen samples from three fishes in each tank removed through proper dissection process biochemical and real-time PCR analysis of some immune-related genes. The samples were kept at -80 °C and stored until used for RNA purification. Fish bled from caudal peduncle, and blood was transferred immediately to tubing and after centrifugation, serum was removed and frozen at -80 °C until use. Total protein, albumin, globulin, SOD, CAT, and GPx following AST and ALT enzymes were analyzed (Labh, 2020) using available kits.

3.3. Blood morphology:
Blood collected from the subsequent puncture caudal vessels via the lateral and ventral approach techniques employing a syringe with a 23 G needle. MS222 used for anesthesia (30 seconds) before collecting the blood sample.The blood collected in Li-heparin 2 ml containers for every fish. The ventral approach consisted of placing the needle tip at the ventral median line and within the middle portion of the fishtail peduncle. It slightly inserted the needle cranial and caudal until the tip reached the vertebras. The caudal vessels' lateral approach consisted of positioning the needle just below the receptor within the tail's middle area. The needle was oriented cranially, at a 45° angle and inserted slightly below the vertebras.

3.4. Expression analysis of some immune genes:
Immune-related genes like Interleukin IL-6, IL-8, IL-10, and TNF-α relatives to β-actin genes analyzed using standard procedures gene expression studies. Isolation of total RNA, cDNA synthesis, and real-time PCR process completed using standard procedures and available kits for gene expression analysis. The sequences of primers and also the real-time PCR program are presented in table 2.

Table 2: Primers used in real-time PCR during gene expression analysis.

Genes	Primer sequence	Accession no.	Product length (bp)
IL-6	F: 5' ACTCCCCTCTGTCACACACC 3' R: 5' GGCAGACAGGTCCTCCACTA 3'	DQ866150	91
IL-8	F: 5' AGAATGTCAGCCAGCCTTGT 3' R: 5' TCTCAGACTCATCCCCTCAGT 3'	AJ279069	69
IL-10	F: 5' CGACTTTAAATCTCCCATCGAC 3' R: 5' GCATTGGACGATCTCTTTCTTC 3'	AB118099	70
TNF-α	F: 5' TGGAGGGGTATGCGATGACACCTG 3' R: 5' TGAGGCCTTTCTCTCAGCGACAGC 3'	AJ249755.1	116
β-Actin	F:5' TCACCCACACTGTGCCCATCTACGA 3' R:5' CAGCGGAACCGCTCATTGCCAATGG 3'	AC006483.3	295

4. Statistical analysis:
The results subjected to analysis of variance (ANOVA) followed by the least significant differences (Tukey) test. Correlation coefficients were significant with $P < 0.05$.

RESULTS AND DISCUSSION

1. Effects of MLM on the growth of trout:
Higher percent weight gain and SGR and lower FCR observed in the M20 diet-fed group (Table 3).

Survival percentage was cent percent in all the treated and control diet-fed group. Now days, plant sources are wont to replace the protein in organic fertilizer and soybean flour, either partially or totally and Moringa leaf has been widely studied as another protein source in fish diet and seems to be a capable protein source (Siddhuraju and Becker, 2003, Labh *et al.,* 2019, Agnihotri, 2019; Singh, 2019). Moringa leaf can partially replace conventional diets in the growth performance of Nile tilapia (*Oreochromis niloticus* L.) (Richter *et al.,* 2003), and the practical fish feed has recently been a locality of focus in aquaculture nutrition research (Labh *et al.,* 2015).

Table 3: Rrainbow trout fed different doses of MLM and growth performance after 90 days of trial.

Growth	M0	M10	M20	M30	M40
In Wt	6.57± 0.56	6.57± 0.56	6.57± 0.56	6.57± 0.56	6.57± 0.56
FWt	23.13 ± 0.32	24.05± 0.11	29.52± 0.23	26.95± 0.16	24.06± 0.51
WG	16.56 ± 0.19	17.48± 0.16	22.95± 0.23	20.38± 0.36	17.49± 0.11
WG%	252.05±0.54	266.05±0.57	349.31±0.68	310.19±0.57	266.21±0.13
SGR	1.13±0.27	1.167±0.33	1.35±0.23	1.27±0.19	1.168±0.11
FCR	1.76±0.13	1.53±0.15	1.33±0.11	1.47±0.17	1.61±0.14
S (%)	100	100	100	100	100

In Wt = initial weight, FWt= final weight, WG%=percent weight gain, SGR= specific growth rate, FCR=feed conversion ratio and S(%)=survival rate.

During this study, fish feed included MLM, which is assumed to be useful for the healthy growth of fish, and after the 90 days feeding trial, the endless growth trend observed with no differences between the treated groups but with the treated groups significantly different from the control group. A similar result found when Tilapia fed with a diet containing MLM at 100 g/kg, 130 g/kg, and 150 g/kg replacement with fishmeal-based dietary protein (Richter *et al.,* 2003). These results contrast with Afuang *et al.* (2003), who found that diets supplemented with methanol-extracted MLM containing 11, 22, and 33 g kg-1 failed to affect the expansion of Nile Tilapia *Oreochromis niloticus*. Using raw moringa leaf within the diets for Tilapia showed that the provender protein replacement at a rate of 10% failed to affect the expansion performance (Richter *et al.,* 2003). The present results are similar to (Karpagam and Krishnaveni, 2014), who found a significant increase in weight and specific rate of growth in *Oreochromis mossambicus* fed *Moringa oleifera* leaves as growth promoter at 5% concentration supplemented feed.

2. Effects of MLM on biochemical performances:

In the biochemical performance, significantly higher ($P < 0.05$) total protein contents, albumin, and globulin recorded (Table 4) in all the treated group, and enzyme-like SOD, CAT, and GPx found significantly higher ($P < 0.05$) in M20 diet-fed group. AST and ALT enzyme in the treated group compared to the control group was lower, and the minimum was in the M20 diet-fed group (Table 5). Similarly, four immune-related genes (IL-6, IL-8, IL-10, and TNF-α) studied within the spleen of rainbow trout fed five different levels of MLM diets for 90 days. Significantly higher ($P <$ 0.05) IL-6 (37.98±0.23), IL-8 (31.81±0.23), IL-10 (24.66±0.13), and TNF-α (28.16±0.12) observed compared to the β-actin gene (24.47±0.17) during immune organic phenomenon studies of rainbow trout fed MLM up to 90 days (Figure 2).

Table 4: Rrainbow trout fed different doses of moringa leaf meal (MLM) and protein profile after 90 days of trial.

Protein	M0	M10	M20	M30	M40
TSP	3.22	4.48	5.75	7.27	5.01
ALB	1.41	1.46	1.51	1.61	1.56
GLO	1.81	3.02	4.24	5.66	3.45
A/G	0.78	0.48	0.36	0.28	0.45

TSP=Total serum protein, ALB=albumin, GLO=globulin, A/G=ratio of albumin and globulin.

Table 5: Rrainbow trout fed different doses of moringa leaf meal (MLM) and enzyme profile after 90 days of trial.

Enzyme	M0	M10	M20	M30	M40
SOD	78.22	80.483	82.74	87.26	85.72
CAT	94.41	96.45	98.51	102.61	100.96
GPX	79.78	81.23	82.68	85.58	84.24
AST	45.84	43.56	41.29	36.74	39.16
ALT	37.93	35.32	32.75	30.08	27.71

SOD=Superoxide dismutase., CAT=Catalase, GPx=Glutathione peroxidase., AST= Aspartate Aminotransferase, ALT=alanine aminotransferase

The present experiment indicates total serum protein, albumin, and globulin contents found significantly higher ($P < 0.05$) in all the MLM diet treated groups compared to control. The treated diet fed fish compared to manage, and the better result was in the M20 food fed to fish. Moringa leaves have a comparatively high crude protein content, which varies from 25% (Makkar and Becker, 1996) to 32% (Soliva *et al.,* 2005). A high proportion of this protein is hypothetically available for digestion thanks to a high percentage of pepsin soluble nitrogen (82-91 %) and low balance (1-2%) of acid detergent insoluble protein (Makkar and Becker, 1996).Using solvent extracted MLM, the inclusion level may be triple to 33% without a significant effect on fish performance (Afuang *et al.,* 2003). Nile tilapia (*O. niloticus*) fed tea diet also enhanced the protein contents in fish serum (Abdel-Tawwab *et al.,* 2010).

Like moringa lapsi fruits, *Choerospondias axillaris* fed to rainbow trout found better antioxidant activities (Labh *et al.,* 2019; Shakya and Labh, 2019). The dose of MLM increases within the diet enzyme-like SOD, CAT, and GPx found significantly higher compared to manage the diet-fed group. The antioxidant complexes may protect cells against the destructive impacts of ROS, hydroxyl radicals, and proximities (Labh *et al.,* 2019). The cells are sensitive to antioxidant because of the high percentage of PUFA in their plasma membranes. They suggest that MLM could have an on the spot (antioxidant) effect in antioxidant defense (Crespy and Williamson, 2004). Decreasing trends observed in AST and ALT contents among the treated compared to control diet-fed group. Other studies have also shown reductions of serum ALT and AST in animals treated with an extract of MLM (Gioacchini *et al.,* 2010). The elevated levels of enzymes (AST & ALT) are an indication of their increased entrance in serum from damaged liver cells (Huang *et al.,* 2008), with injury identified using enzymes as hepatic markers (Amar *et al.,* 2004). Studies have shown that the probabilities of the damaged liver having the ability to heal reduced within the acute phase, which frequently results in chronic diseases with complications (Chu and Juneja, 1997).

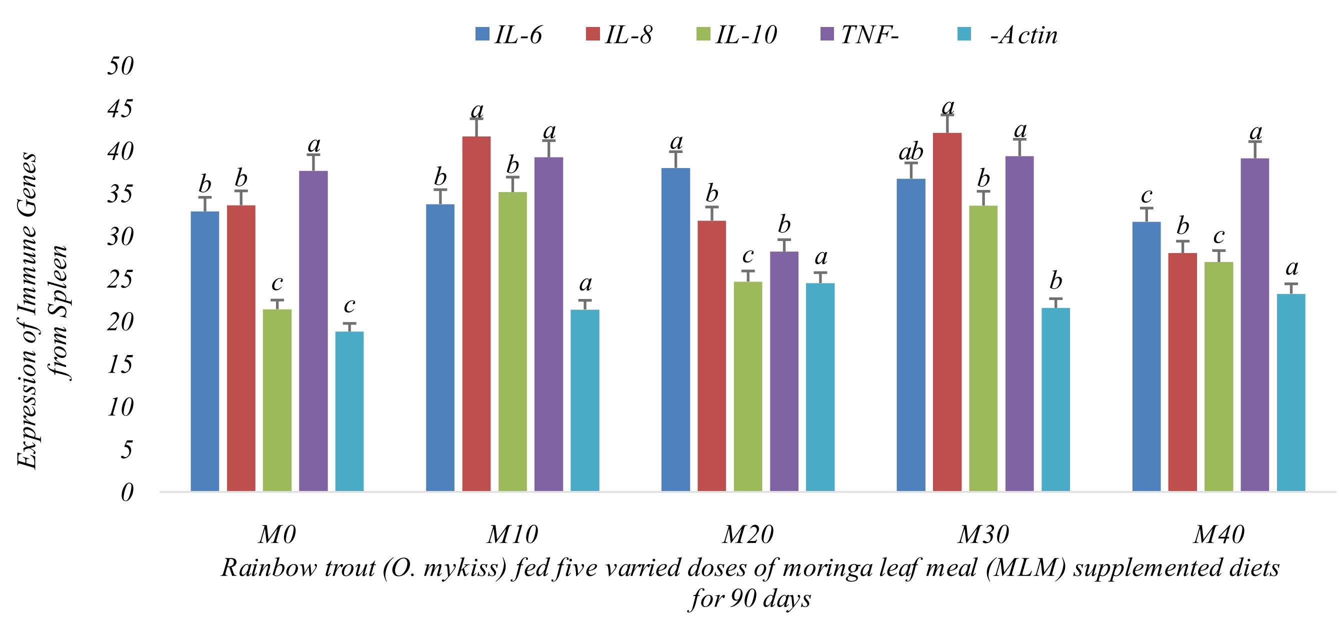

Figure 2: Interleukin (IL-6), Interleukin (IL-8), Interleukin (IL-10), and Tumor Necrosis Factor (TNF-α) genes relative expression levels to β-actin determined by real-time PCR in the spleen of rainbow trout fed five varied MLM supplemented diets for 90 days. Data represented as means ± standard error (n = 15).

3. Effects of MLM on some immune gene expression of trout:

Labh (2020) explains the immune responses that involve a broad range of molecules, receptors, and cells from different aspects. The immune modulation of mRNA expression related to cytokine genes is also a valuable tool. Similarly, Costa *et al.* (2011) justified IL-6, IL-8, and IL-10 genes, which play a vital role in the host response to microbial invasion. In the present study, the IL-6 gene regulated high at M20 in spleen tissues, and the effect of moringa leaf meal has dose-dependent. Similarly, the IL-6 organic phenomenon up-regulated in rainbow trout is noticed following *in vivo* stimulation with β-glucan (Raida and Buchmann, 2008). IL-8 may be a chemokine produced by numerous cell types like macrophages/monocytes, epithelial cells, neutrophils, fibroblasts, and endothelial cells upon infection or stimulated by cytokines like IL-6 and TNF-α (Jimenez *et al.,* 2006). A significant increase ($P < 0.05$) in IL-8 shown in fish spleen fed with M20 and M30, respectively.

Interleukin (IL-10) is the regulatory cytokine that proves immune function. The most feature of IL-10 seems to be regulation of the inflammatory response, thereby minimizing damage to the host induced by an excessive response (Watanuki *et al.,* 2006). IL-10 inhibits the effect of pro-inflammatory cytokines and controls cytokine synthesis (Raida and Buchmann, 2008). The results obtained for the IL-10 gene obtained for IL-IL-6 and IL-8. Expression levels of IL-10 also found following the administration of different stimulants like *Spirulin*a (Watanuki *et al.,* 2006) and Lapsi (Labh, 2020).

Tumor necrosis factor-alpha or TNF-α is a vital component during the onset of early inflammatory events. TNF-a is synthesized by various cell types upon stimulation with endotoxin, inflammatory mediators, or cytokines like IL-1 and, in an autocrine manner, upon stimulation by TNF itself (Faheem *et al.,* 2020). During this study, MLM induced significantly higher TNF-α organic phenomenon levels in spleen tissue at M20 compared to the control M0, implying involvement through this mediator of response. The effects of MLM were tissue-specific and dose-dependent. TNF-α organic phenomenon level also up-regulated in Common carp spleen that received a high dose of Astragalus polysaccharides (APS) (Hoseinifar *et al.,* 2019). Feeding nucleotide-supplemented diets to turbot also increased pro-inflammatory cytokines and TNF-α (Low *et al.,* 2003).

CONCLUSION

Incorporation of a moringa leaf meal (MLM) supplement into the fish diet may result in immune-related gene expression in the dose and tissue-dependent manners. The results show that dietary supplementation of 20% MLM is ideal for effective growth performance; enhance the antioxidant activities and immune responses in rainbow trout. More studies are recommended to research the consequences of the high level of moringa leaves supplemented diets on fish performance.

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