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THE IMPACT OF DIETARY INCLUSION OF AMARANTH MEAL ON HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF BLOOD AND HISTOPATHOLOGICAL CHANGES IN LIVER OF RAINBOW TROUT*

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Abstract

The effect of dietary inclusion of amaranth meal on hematology and blood biochemistry, of rainbow trout *Oncorhynchus mykiss* was examined. The fish (mean length 35.2 ± 0.6 cm; mean weight 524.8 ± 28.5 g) were divided into three groups: a reference group (RF) fed commercial trout pellet and two experimental groups (EF5 and EF10) fed feeds containing 5.0% and 10.0% of amaranth meal respectively. Determined indices covered: packed cell volume, red blood cell count, hemoglobin concentration, mean cell volume, mean cell hemoglobin concentration, mean hemoglobin content, inorganic phosphates, total proteins, albumins, globulins, ammonia, triacylglycerols, glucose, and the activity of creatine kinase, alkaline phosphatase and aspartate aminotransferase. Supplementation of feed with amaranth meal significantly raised levels of blood glucose, cholesterol, total protein, ammonia, creatinine and aspartate aminotransferase activity in trout blood.

These results indicate that inclusion of amaranth meal in extruded diets for rainbow trout can get negative effect on liver and blood biochemistry profile.

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Introduction

The replacement of fishmeal as a major protein source with plant origin components is challenging the sustainability of aquaculture industry (VILHELMSSON et al. 2004). There is a general interest in increasing the level of plant protein ingredients in feeds for farmed fish. (GATLIN et al. 2007). Many researchers in recent years investigated influence of vegetable components on fish growth, liver and intestinal histology (HANSEN et al. 2006, 2007, ESCAFFRE et al. 2007, LANSARD et al. 2009, BORQUEZ et al. 2010, MURASHITA et al. 2013, RANDALL et al. 2013), hematological and blood biochemical profile (YAMAMOTO et al. 2010, MURASHITA et al. 2013, TUSCHE et al. 2013, LÓPEZ et al. 2015).

The main problem with plant components is anti-nutritional factors (ANFs) impacting salmonid fish yield, by means of decreased digestion and reduced utilization of proteins followed by decreased growth rates (MOYANO et al. 1991, KROGDAHL et al. 1994), feed intake and decreased nutrient absorption (FRANCIS et al. 2001). Some researchers reported impact of ANFs on fish hematology and blood biochemistry (SHAFAEIPOUR et al. 2008, IWASHITA et al. 2009, KUMAR et al. 2010). Protease inhibitors, phytic acid, lectins, gossypol, glucosinolates and saponins are some of the most common ANFs in plant ingredients used in fish feeds (TACON 1997, KROGDAHL et al. 2010). The effectiveness of common processing techniques such as dry and wet heating, solvent extraction, and enzyme treatment in removing ANFs from vegetable raw materials was discussed in many papers (FRANCIS et al. 2001, KUMAR et al. 2010). However, processing is usually expansive and/or decreases nutritional value of raw materials.

Balanced amino acid profile with high level of lysine makes amaranth (*Amaranthus cruentus*) seeds attractive protein source for fish (PEDERSEN et al. 1987). However, chemical analysis of amaranth showed low level of ANFs: phytic acid and saponins (ESCUDERO et al. 2004). Previous studies have shown, high level of amaranth meal (level at 5–10%) summplentation induced negative influence on the growth performance and liver condition. (NIEWIADOMSKI et al. 2016).

We suppose that the addition of amaranth flour may have a negative effect on the health status of rainbow trout. Current research was at the aim of the study was the assessment of effect of dietary inclusion of amaranth meal on health status – hematology, blood biochemistry and liver histology of rainbow trout *Oncorhynchus mykiss*.

Materials and Methods

Fish, feeding, experimental system, diet preparation

Rainbow trout with initial mean length of 35.2 ± 0.6 cm and mean body weight of 524.8 ± 28.5 g were used in the experiment. Fish (n = 144) were randomly divided into three groups: a reference group (RF) fed commercial trout pellet and two experimental groups (EF5 and EF10) fed pellets that contain 5.0% and 10.0% of amaranth meal respectively. Fish were distributed in 9 tanks (3 groups in triplicates; n = 16 for each replicate). Experimental conditions have been described by NIEWIADOMSKI et al. (2016). Fish were sampled after 21 days of feeding. Ingredients and nutrients composition of the experimental diets are presented in Table 1.

Table 1
Ingredients and chemical composition of experimental diets (following NIEWIADOMSKI et al. 2016)

Ingrediens [%]	EF5	EF10	RF*
Fishmeal	44.25	44.25	NA
Wheat flour	20.31	20.31	NA
Soybean meal	15.00	10.00	NA
Amaranth meal	5.00	10.00	NA
Fish oil	6.24	6.24	NA
Soybean oil	6.00	6.00	NA
Vitamin premix[1]	2.00	2.00	NA
Mineral premix[2]	0.10	0.10	NA
Choline	0.50	0.50	NA
Ascorbic acid	0.50	0.50	NA
Chromic oxide	1.00	1.00	1.00
Chemical composition [%]			
Dry matter	94.27	95.02	93.51
Crude protein	41.01	40.62	42.26
Crude fat	11.95	13.45	13.70
Crude ash	10.44	10.43	8.52
Crude fibre	3.27	3.25	3.22
Chromic oxide	1.00	1.00	1.00
Gross energy [MJ kg^{-1}]	18.04	15.41	17.42

* Data not available

[1] Witamin premix (IU kg^{-1} or mg kg^{-1} dry diet): witamin A – 15 000 UI kg^{-1}; witamin D – 6000 UI kg^{-1}; witamin E – 15; witamin C – 70; witamin B_1 – 0.8; witamin B_2 – 3.0; witamin B_6 – 1.50; witamin B_{12} – $8 \cdot 10^{-3}$; witamin K – 1.5; biotin – 2.5.

[2] Mineral premix (mg kg^{-1} dry diet): calcium – $25 \cdot 10^3$; phosphorus – $27 \cdot 10^3$; sodium – $18 \cdot 10^3$; magnesium – $2 \cdot 10^3$; mangan – 720; iron (II) – 400; copper – 127; zinc – 800; iodine – 23.

Experimental feeds were extruded with a co-rotating twin screw extruder (METALCHEM, Poland) equipped with a Ø 4.5 mm pellet stencil.

Chemical analysis

The content of the basic chemical components in feed (dry matter, crude protein, crude fat, ash) was determined in accordance to standard methods (AOAC 2016). Dry matter was determined by drying in an oven at 105°C for 24 h. Total protein was determined by Kjeldahl's method and crude fat by Soxhlet's method.

Hematology

Before the blood sampling, fish were caught, immediately anaesthetized with propofol – 7 mg dm^{-3} (GOMUŁKA et al. 2014). Blood was sampled with a syringe covered with heparin lithium salt from caudal vessels. Hematological indices were determined according to SVOBODOVA et al. (1991) and covered: hematocrit (PCV), hemoglobin concentration (HB), red blood cell count (RBC), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), mean hemoglobin content (MHC).

Biochemistry indices

Blood samples were centrifuged at 12 000 g for 30 s and frozen. Plasma samples were analyzed with a Catalyst Dx Chemistry Analyzer (Idexx Lab; USA) using dedicated test slides (custom panels). The following biochemical measurements were performed: albumins (ALB), globulins (GLOB), total protein (TP), ammonia (NH_3), glucose (GLU), triacylglycerols (TAG), cholesterol (CHOL), creatinine (CR) and the activity of amylase (AMS), lipase (LIP), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Each plasma sample was thawed only once at room temperature and all of the above measurements were performed at once to eliminate multiple freezing/thawing cycles.

Liver histology

Randomly collected liver samples (n = 9 per group) were fixed in Bouin's fluid, dehydrated in ethanol, cleared in xylene, embedded in paraffin blocks, and then sliced into 4–5 µm sections with a RM 2155 rotational microtome (LEICA Microsystems, Wetzlar, Germany). Cross-sections of tissues were stained with haematoxylin and eosin (ZAWISTOWSKI 1986). The number of hepatocytes was established in a field with a surface area of 2500 μm^2 (50×50 µm). These measurements were performed with a LEICA DM 3000 light microscope and LEICA QWin Pro micro image

analysis software (LEICA Microsystems AG, Heerbrugg, Switzerland). For each fish, the diameters of 100 hepatocytes (HD) and their nuclei (HND) were measured under the light microscope equipped with computer analysis software. Nuclear-cytoplasmic index (NCPI) was calculated as follow: $NCPI = HND \cdot HD^{-1}$.

Statistical analysis

Normality of data distribution was tested by Shapiro-Wilk test and variance homogeneity by Leven's test. When above assumptions were met, differences between means were analysed using ANOVA and *post hoc* Tuckey's test (TT). For the others, Kruskal-Wallis ANOVA and Dunn's test (DT) were used. Results were analysed with Statistica 12.0 (Statsoft, USA) software at significance level $P \leq 0.05$.

Results

Haematology

No significant differences were found between experimental groups in RBC, Hb, PCV, MCV and MHC (TT, $P > 0.05$). Significantly higher values of MCHC were determined in EF10 group (TT, $P < 0.05$) when compared to the reference group (Table 2).

Table 2

Hematological indices of *Oncorhynchus mykiss* fed experimental diets formulated with amaranth meal

Specification	RF	EF5	EF10
RBC [T l^{-1}]	$0.74^a \pm 0.15$ (0.44–1.01)	$0.66^a \pm 0.15$ (0.36–0.97)	$0.67^a \pm 0.18$ (0.34–0.99)
Hb [g l^{-1}]	$97.2^a \pm 8.9$ (74.6–115.8)	$98.9^a \pm 10.5$ (77.7–117.5)	$102.1^a \pm 10.0$ (84.5–119.5)
PCV [1]	$0.34^a \pm 0.06$ (0.22–0.47)	$0.35^a \pm 0.03$ (0.27–0.41)	$0.33^a \pm 0.04$ (0.25–0.39)
MCV [fl]	$449^a \pm 74$ (333–554)	$565^a \pm 157$ (378–958)	$518^a \pm 175$ (253–1072)
MHC [pg]	$138^a \pm 37$ (93–251)	$159^a \pm 47$ (95–276)	$163^a \pm 52$ (101–303)
MCHC [pg fl^{-1}]	$285^a \pm 33$ (222–331)	$284^a \pm 34$ (214–348)	$319^b \pm 50$ (251–427)

Results are presented as mean ± SD (range). Number indexes show columns with significantly different results ($P \leq 0.05$)

Blood biochemistry profile

Results of biochemical blood analysis are presented in detail in Table 3. No significant differences were found between experimental groups in the case of TAG, ALB, GLOB, AMS, LIP, ALP level (TT, $P > 0.05$). In the other

biochemical indicators not mentioned above, a statistically significant increase was observed in relation to the reference group (TT, $P < 0.05$).

Table 3

Blood biochemical parameters of *Oncorhynchus mykiss* fed experimental diets formulated with amaranth meal

Specification	RF	EF5	EF10
GLU [mmol dm^{-3}]	5.73^{a} ± 2.06 (2.97–9.72)	8.43^{b} ± 3.51 (3.01–17.35)	7.38^{ab} ± 2.60 (2.26–13.86)
TAG [µmol dm^{-3}]	2.47^{a} ± 0.86 (1.31–4.11)	2.94^{a} ± 0.85 (1.38–4.24)	2.72^{a} ± 0.97 (1.04–4.24)
TP [g dm^{-3}]	45.0^{a} ± 7.3 (34–60)	51.5^{b} ± 9.0 (37–74)	50.1^{ab} ± 8.7 (37–66)
ALB [g dm^{-3}]	18.5^{a} ± 2.9 (15–25)	19.0^{a} ± 2.4 (12–23)	19.1^{a} ± 2.2 (16–23)
GLOB [g dm^{-3}]	26.5^{a} ± 5.0 (19–55)	33.0^{a} ± 8.8 (22–55)	31.9^{a} ± 7.6 (21–47)
CHOL [mmol dm^{-3}]	4.85^{a} ± 1.02 (3.34–7.39)	5.88^{b} ± 1.62 (3.73–8.76)	5.86^{b} ± 1.32 (3.82–7.76)
NH_3 [µmol dm^{-3}]	126^{a} ± 54 (37–220)	184^{a} ± 80 (49–333)	207^{b} ± 79 (71–373)
CR [mmol dm^{-3}]	13^{a} ± 4 (9–19)	89^{b} ± 36 (35–164)	123^{b} ± 83 (33–330)
AMS [U l^{-1}]	1279^{a} ± 369 (506–2019)	1240^{a} ± 377 (490–1841)	1176^{a} ± 341(641–1736)
ALP [U l^{-1}]	193^{a} ± 86	254^{a} ± 100	243^{a} ± 123
LIP [U l^{-1}]	153^{a} ± 38 (84–232)	176^{a} ± 110 (95–647)	154^{a} ± 30 (106–203)
AST [U l^{-1}]	39.9^{a} ± 16.7	58.3^{b} ± 20.1	56.3^{b} ± 18.6

Results are presented as mean ± SD (range). Number indexes show columns with significantly different results ($P \leq 0.05$)

Liver histology

The addition of amaranth meal affects the size of hepatocytes and the NCPI is statistically significant increase (TT, $P < 0.05$) in experimental groups (Table 4). The representative histological picture of liver sections from the fish fed with experimental and reference diet are shown on Figure 1.

Table 4

Measurements of hepatocytes of *Oncorhynchus mykiss* fed experimental diets formulated with amaranth meal

Specification	RF	EF5	EF10
HD [µm]	14.60^{a} ± 41.99	16.88^{b} ± 0.99	17.63^{b} ± 1.44
HND [µm]	5.86^{a} ± 0,59	5.81^{a} ± 0.21	6.35^{a} ± 0.06
INCP [1]	0.41^{a} ± 0.03	0.34^{b} ± 0.01	0.36^{b} ± 0.03

Results are presented as mean ± SD. Different superscripts means significantly different results in rows ($P \leq 0.05$). HD – hepatocyte diameter; HND – hepatocyte nucleus diameter; NCPI – nucleo-cytoplasmatic index

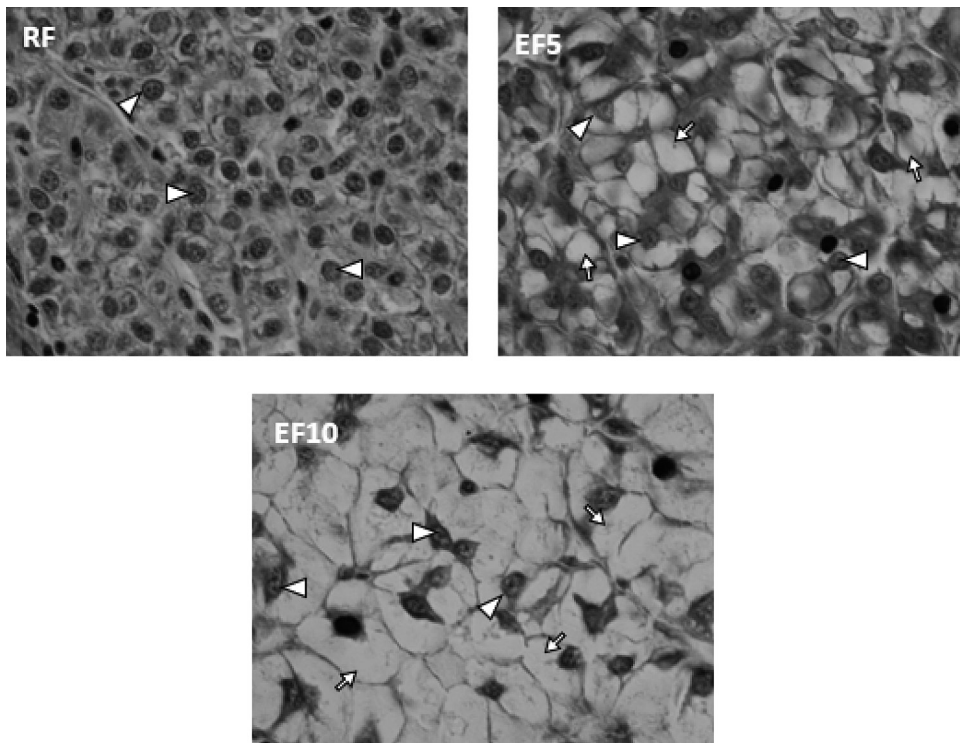

Fig. 1. Representative picture of liver sections of *Oncorhynchus mykiss* fed reference and experimental diets formulated with amaranth meal content. H & E. Reference feed (RF), experimental feeds (EF5 and EF10), hepatocyte nuclei (arrow heads), vacuole (arrows). Photographs were taken at approximately 40x.

Discussion

The hepatosomatic index (HSI) is among the most popular indices used to identify possible liver disorders (VAN DER OOST et al. 2003). The HSI in experimental fish (1.96 and 2.08 for EF5 and EF10 feeds respectively) was much higher compared to values reported for rainbow trout fed with soya meal based diet (1.18) (ØVERLAND et al. 2009) and lupine meal based diet (1.1) (BORQUEZ et al. 2011). This increase was probably caused by fat and/or glycogen accumulation inside hepatocytes (Figure 1), what was followed by the increase of mean hepatocyte diameter and resulted in growth of liver mass. GÜMÜŞ AND İKIZ (2009) found that increasing NFE level in feeds caused the HSI increase in rainbow trout. Although the NFE levels in reference and both experimental diets were similar (25,81% and 27.60% and 27.70% respectively), our results seems to be in agree with above finding as apparent digestibility of NFE was much lower in refer-

ence diet comparing to EF5 and EF10 feeds (47.4% and 75.5% and 78.4% respectively) (NIEWIADOMSKI et al. 2016). Higher digestibility of amaranth meal NFE can be probably addressed to small-particle size and easy to digest amaranth starch (ECKART et al. 2002). However, MURASHITA et al. (2013) recorded similar histopathological changes in liver of trout fed feed with soya meal-based diets.

High digestibility of NFE was probably the cause of hyperglycaemia found in the experimental fish. Usually, glucose level in blood plasma of rainbow trout ranged between 4.25 and 5.73 mmol dm^{-3} (FIGUEROA et al. 2000, SANTIN et al. 2013). In our experiment, glucose level reached 7.38 and 8.43 mmol dm^{-3} in EF5 and EF10 respectively. These level is much higher than results reported for fermented soya meal (5.7–6.6 and 6.2–6.6 mmol dm^{-3}) and soya protein concentrate (3.34–5.67 mmol dm^{-3}) diets respectively (YAMAMOTO et al. 2010, 2012, KUMAR et al. 2010, and MURASHITA et al. 2013). YAMAMOTO et al. (2007) and TUSCHE et al. (2013) reported similar results for trout fed soya protein concentrate (7.2–7.6 mmol dm^{-3}) and potato protein concentrate based diets (7.4–9.9 mmol dm^{-3}) respectively. However, we are aware that the very high glucose level could be the result of our experiment design. We did not stop feeding 24 hours before blood sampling because we examined apparent digestibility of nutrients in the same experiment (results are reported in NIEWIADOMSKI et al. 2016). From the other hand, blood glucose level in the reference group (5.73 mmol dm^{-3}) was not altered.

Morphological changes in the liver were accompanied by 1.5-fold increase of AST serum activity in experimental groups. AST similarly to ALP and ALT belongs to cytoplasmic intracellular enzymes and it appears in blood plasma due to a damage to cell membranes. Such increase of activity can be a result of parenchymal organs damage or breakdown of red blood cells (RACICOT et al. 1975). However, as we did not record any differences in RBC or PCV between reference and experimental groups, we believed that the AST activity increase is the sequel of pathological processes observed in fish liver. Moreover, increase of ALP activity, although not significant, suggest increased metabolic burden of the liver (KUMAR et al. 2010) and support above statement.

From the other hand, 7.9 to 9.5-fold higher level of CR in fish fed with experimental diets (89 and 123 μmol dm^{-3} for EFD5 and EF10 respectively) when compared to reference group (13 μmol dm^{-3}) suggest that some kidney failure also occurred. According to CHAROO et al. (2013) high CR level is an indicator of impaired renal function. Moreover, mean CR level was correlated to mean NH_3 level ($r = 0.9996$; $p < 0.05$) which was also significantly higher in experimental fish blood.

Total protein level in trout blood in experimental groups (50.1–51.5 g l^{-1}) was higher than those reported by YAMAMOTO et al. (2007, 2010) for fermented soybean meal (30–36 g l^{-1}), KUMAR et al. (2010) for jatropha flour (38–41 g l^{-1}), MURASHITA et al. (2013) for soybean meal (33–36 g l^{-1}) and TUSCHE et al. (2013) for potato concentrate (33.3–36.8 g l^{-1}). According to BANAEE et al. (2011) there is a close relationship between the rate of protein synthesis in the liver and the concentration of total blood protein. However, such high results suggest some pathological reasons. SHAMOUSHAKI et al. (2012) studied the EDTA toxicity to rainbow trout. They found similar TP levels in trout exposed to 1.4 g l^{-1} to 2.1 g l^{-1} of EDTA (52.3 g l^{-1} to 58.3 g l^{-1} respectively). YILMAZ et al. (2015) found TP levels as high as 100.2 g l^{-1} to 106.3 gl^{-1} in trout orally exposed to carvacrol. These authors addressed the increased level of TP to the increase in innate immune response of fish. However, in their study TP level in the control group was also extremally high (87.2 g l^{-1}).

Many authors found lowered CHOL level in fish fed feeds based on plant origin raw material (ROMARHEIM et al. 2006, IWASHITA et al. 2008, YAMAMOTO et al. 2007, 2010, KUMAR et al. 2010). Values of CHOL recorded in experimental fish blood plasma (5.86–5.88 mmol dm^{-3}) in our experiment were much higher than those reported by SHAFAEIPOUR et al. (2008), IWASHITA et al. (2008), YAMAMOTO et al. (2010, 2012), MURASHITA et al. (2013) for rape (2.10–2.83 mmol dm^{-3}), soybean (2.04–2.32 mmol dm^{-3}), fermented soybean (2.74– 2.96, 2.93–3.41 mmol dm^{-3}) and modified soybean (3.22–4.09 mmol dm^{-3}) meals respectively. We suppose that such high increase of blood CHOL level in experimental fish was a result of cholesterol synthesis in the liver from the squalene which is present in amaranth seeds. Squalene is the precursor of cholesterol. The squalene level in amaranth meal is about 6.23% (ESCUDERO et al. 2004). The increase of cholesterol synthesis was found in hamster fed with amaranth supplemented feed (MENDONÇA et al. 2009).

According to DENG et al. (2013) the blood cholesterol level is built by exogenous cholesterol supplied in feed and cholesterol synthetized *de novo* in the liver. The authors suggested that in case of excessive supply of cholesterol for a longer time, fish can inhibit endogenous cholesterol production. SHAFAEIPOUR et al. (2008) obtained the reduction of CHOL level in trout blood plasma after 112 days of feeding with experimental canola diets when compared to results obtained after 56 days of feeding. We can expect that longer feeding with amaranth meal feed can result in lower cholesterol blood level.

KROGDAL et al. (2010) found that despite heat treatment many ANFs (for example saponins) are not eliminated from feeds. Saponins present in

both soya and amaranth meals can impact some blood parameters including CR, TP and NH_3 causing liver and kidney (PISARIKOVA et al. 2006) or gills impairment (FRANCIS et al. 2001)

Conclusions

However, one should take into account that the experiment was relatively short. Thus, our results should be considered as a preliminary study. These results suggest that inclusion of amaranth meal in extruded diets for rainbow trout can get negative impact on functional status on liver and kidney of rainbow trout. We assume that the obtained results were influenced by ANFs derived from soybean meal and amaranth meal. Next experiment, describe to the effect of amaranth meal feeding on fish with amaranth meal as the only one source of potential ANFs.

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