The Influence of Some Phytobiotics on Haematological and Some Biochemical Indices at *Oreochromis Niloticus–Linnaeus, 1758*

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Abstract
The aim of this research was to evaluated the influence of some phytobiotics on haematological profile, leukocyte reaction and some biochemical indices at *Oreochromis niloticus* species, reared in a recirculating aquaculture system. This experiment was conducted six weeks. The experimental variants were: V1–control; V2–1% *Rosmarinus officinali*/kg feed; V3–1% *Hippophae rhamnoides*/kg feed and V4–1% *Zingiber officinale*/kg feed. Blood was analyzed using standard techniques. At the end of the experiment the following parameters were determined: RBCc ($x10^6$cells/µL), Hb (g/dL), PVC (%), MCV (μm^3), MCH (pg), MCHC (g/dL), TP (g/dL), GLU (mg/dL), cortisol (ng/mL), lysozyme activity (U/mL), absolute number of blood cells ($x10^3$ cells/µL) and leukogram (%). The results showed that the administration in feed of some phytobiotics lead to signifiant differences ($p<0.05$) of following parameters: RBCc ($x10^6$cells/µL), MCV (μm^3), glucose (mg/dL), lysozyme activity (U/mL), monocyte (%) and in absolute number of leukocytes, lymphocytes and monocytes. In conclusion, due to decreasing of RBCc, PVC, Hb, MCHC, cortisol, GLU and due to normal concentration of TP, we can say that the administration of sea buckthorn and ginger, but even rosemary administration, in diet improves the physiological status at *Oreochromis niloticus* species.

Keywords: haematological profile, leukogram, lysozyme activity, *Oreochromis niloticus,* phytobiotics, recirculating aquaculture system

1. Introduction

Tilapia (including all species) is the second most important group of farmed fish after carps, and the most widely grown of any farmed fish [1, 2]. Due to intensive culture practices for the increased production, external stressors (diseases, transport handling, water quality, etc.) continue to pose a serious threat to aquaculture industry, leading in specially to physiological changes [3-6].
The administration of medicinal plant in fish feed is considered as a modern and promising alternative to antibiotics and vaccines as a prophylactic measure in intensive aquaculture.

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Various products derived from medicinal plants have been studied concerning their ability to preventing diseases in a variety of fish species [7, 8], establishing that they present immunostimulatory properties.
Analysis of haematological profile is an important tool that can be used as an effective and sensitive index to monitor in fishes the physiological and pathological changes.
Usually, evaluation of haematological profile consists in determination of total red blood cell count (RBCc), hematocrit (PVC), hemoglobin concentration (Hb), erythrocyte constants (mean corpuscular volume-MCV, mean corpuscular hemoglobin-MCH, mean corpuscular hemoglobin concentration-MCHC), total white blood cell count (WBCc), white blood cell differential count

by evaluating the blood smears, thrombocytes count [9].
The normal values of the haematological parameters differ greatly from one species to another species. Presence of normal values for number of erythrocytes, leukocytes and thrombocytes plays an important role in oxygen transport, immune function and clot formation [10].
The welfare status of fish can be also analysed with the help of some biochemical blood indices (glucose, cortisol, total proteins) [11].
The levels of cortisol and glucose are considered to be specific indicators of sympathetic activation during stress conditions [12].
On the other hand, some authors claim that serum total proteins is the most importantly indicator of the biochemical nutritional and health status of the fish [13, 14].
Herbal immunostimulants are substances which activate white blood cells (WBC) and may render fishes more resistant to infectious diseases, by the stimulating phagocytic cells as well as complement, lysozyme and antibody responses of fish [15].
The purpose of this study is to investigate the influence of rosemary, sea buckthorn and ginger on haematological profile and some biochemical indices of blood at *Oreochromis niloticus* species, reared in a recirculating aquaculture system.

2. Materials and methods

Experimental design
This experiment was conducted in the research laboratory of Aquaculture, Environmental Science and Cadastre Department from "Dunarea de Jos" University of Galati. This recirculating system was described in our precedent paper [16]. The design of this system consists in four rearing units, with a volume of $1m^3$ each, and a series of water quality conditioning units [17]. The experiment lasted six weeks, from 17.08.2012 to 28.09.2012.
In this research were used a total number of 168 exemplars of Nile tilapia, with an initial average weight of 280.07±54.03 g/fish, that were randomly distributed in four rearing units.
The experimental variants were organized as follows: V1–control, V2–1% rosemary (*Rosmarinus officinalis*)/kg feed, V3–1% sea buckthorn (*Hippophae rhamnoides*)/kg feed and V4–1% ginger (*Zingiber officinale*)/kg feed. These phytobiotics were purchased from a Plafar market, as a powder. Introduction of phytobiotics in feed was performed using an aqueous solution of gelatin with 2% concentration. The feed was sprayed, mixed and then dried at 25ºC.
Fish were fed with SOPROFISH pelleted feed, with 38% crude protein and 7% crude fat. The feed biochemical composition was related by Antache et al., 2013 [2]. Fish were fed four times per day with a daily ration of 2% from fish body weight. At the end of the experiment the individual average weight was 455.43±89.16 g/fish in V1, 446.00±73.95 g/fish in V2, 466.26±65.33 g/fish in V3 and 461.48±71.76 g/fish in V4.

Blood sampling and analysis
Blood sampling has been carried out at the beginning (Vi) and at the ending of experimental periode. Was sampling 3.5 ml of blood at 7 fish from each growth unit. For each sample were used two eppendorf tubes. So, for hematology analyzes was added anticoagulant in eppendorf tubes and for biochemical analyzes was not added anticoagulant. A part of the blood was used for haematological study also for determining glucose, cortisol, total protein and lysozyme activity, and the other part was used for analysis of oxidative stress that is the subject of another study–Antache et al., 2013 [18].
Blood analysis was performed by method used in fish hematology described by Blaxhall, 1973 [19]. This analysis consisted in determination of red blood cells count (RBCc, $x10^6$cells/mm^3), hemoglobin (Hb, g/dl) and hematocrit (PVC, %). The erythrocyte number was determined by counting the erythrocytes from 5 small squares of Neubauer hemocytometer using Vulpian diluting solution. The hematocrit was performed by duplicate using capillary tubes centrifugated for 4 minutes at 13000 rpm in a micro hematocrit centrifuge. The hemoglobin concentrations were measured spectrofotometrically with SPECORD 210 Analytikjena at λ-540 nm, using Drabkin reagent.
Then, using standard formulas described by Ghergariu et al., 1985 [20] and Svobodova, 2001 [21] were calculated the erythrocyte constants: mean corpuscular volume (MCV, μm^3), mean corpuscular hemoglobin (MCH, pg), mean

corpuscular hemoglobin concentration (MCHC, g/dl).

In this experiment was analyzed four biochemical parameters of blood (glucose–mg/dL, cortisol–ng/mL, total protein–g/dL and lysozyme activity–Units/mL). To obtain blood serum, the blood without anticoagulant was centrifuged 10 minutes, at 3500 rotation/min.

Determination of glucose, total protein and lysozyme activity from serum was performed spectrophotometric using the spectrophotometer SPECORD 210 Analytikjena.

Dosage of glucose was made by colorimetric method with o-toluidine, readings were made at 635nm wavelength.

Total protein from serum were determined by Biuret method, the readings was done at a 546nm wavelength.

Lysozyme activity was measured, from serum, based on the turbiometric assay, Enzymatic Activity of Lysozyme Protocol (Sigma, EC 3.2.1.17) [22]. For this test was prepared a substrate, in 66mM Potassium Phosphate Buffer, with 6.24 pH at 25°C, a volume of 0.01% (w/v) suspension of *Micrococcus lysodeikticus* (Sigma, M3770). Lyophilised powder of chicken egg white lysozyme (Sigma, L6876) was used as standard. One unit of lysozyme activity was defined as a reduction in absorbancy of 0.001/min, at a 450 nm wavelength.

Serum cortisol determination was performed using the kit: NovaTec Cortisol–DNOV001 based on competitive immunoenzymatic colorimetric method for quantitative determination of Cortisol in human serum or plasma. Absorption was read at 450nm using an ELISA microwell plate reader.

The relative proportion of each type of white blood cells was obtained by microscopic examination of 200 leukocytes on blood smears (two per eacth fish), using Zeizz Axio Imager microscop and immersion objective (10 oc. X 100 ob.). These were immediately dried, fixed with methanol and then colored with May-Grünwald Giemsa panoptic method (MGG).

The type of leukocytes were determined based on identification characters listed by Svobodova et al., 1991 [23]. Absolute number of circulating blood leukocytes and thrombocytes was determined in comparison with 1000 erythrocytes counted on haemocytometer, per blood volume unit.

Knowing the fact that 2-phenoxyethanol anesthesia had no effect on haematological profile [24], fish were anesthetized with 2-phenoxyethanol (8 mL/40 L of water for 5 minutes) in order to reduce handling stress.

Statistical analysis

The results, of haematological and biochemical parameters, of the experimental groups were statistically analyzed using descriptive statistics and ANOVA test. Programs used were Microsoft Excell 2010 and SPSS Statistics 17.0. The results were presented as mean±standard deviation.

3. Results and discussion

The haematological and some biochemical changes of our researched fish were analyzed in corroboration with the feeding management (using three phytobiotics) which can influence the metabolic processes.

Regarding the haematological and biochemical parameters the results are presented in Table 1.

Table 1. Variation of haematological and biochemical parameters during the experiment

Experimental stage		17.08.2012	28.09.2012			
Experimental variant		Vi	V1	V2	V3	V4
Haematological and biochemical parameters studied (M±SD)	RBCc ($x10^6/\mu L$)^{ab}	1.828±0.12	1.569±0.29	1.937±0.12	1.548±0.31	1.473±0.08
	PVC (%)^{c}	28.40±1.10	29.00±1.79	25.80±3.06	27.80±3.49	30.40±3.26
	Hb (g/dL)^{c}	8.50±0.56	8.95±0.76	7.79±1.25	7.73±0.96	7.89±0.85
	MCV (μm^3)^{ab}	155.91±7.63	193.59±49.32	133.07±12.00	183.99±27.97	206.72±22.47
	MCH (pg)^{c}	47.65±2.35	59.71±15.97	40.21±6.00	52.16±13.08	53.68±5.84
	MCHC (g/dL)^{c}	30.43±1.46	30.82±1.29	30.06±2.26	27.97±2.95	26.14±3.20
	GLU (mg/dL)^{ab}	89.18±7.67	77.54±3.82	95.00±8.27	90.71±7.12	99.71±8.03
	TP (g/dL)^{c}	6.37±0.35	6.59±0.21	6.44±0.42	6.39±0.41	6.86±0.32

"a–significant differences between variants at the end of the experiment ($p<0.05$)

"b"–significant differences between initial and final experimental variants ($p<0.05$)

"c–insignificant differences ($p>0.05$).

At the end of the experiment between experimental variants were obtained significant differences ($p<0.05$) at the level of RBCc ($p=0.04$), MCV ($p=0.02$) and GLU ($p=0.01$), but also between initial value of RBCc, MCV and GLU and final values ($p=0.02$; $p=0.01$; $p=0.01$). Although the results concerning red blood cell count, mean corpuscular volume and glucose presented significant differences between variants, the values obtained falls within the reference range (1.01-7.2 $x10^3$cell/µL [25], 20-214 $µm^3$ [25] or 12.36-528.57 $µm^3$ [26], respectively 22.7-107 mg/dL [25]) for tilapia.

The hematocrit percentage recorded the highest value in variant V4 and the lowest value in V2, but these values fall within the normal range for tilapia, respectively 15-45% [26]. Other researchers have reported for hematocrit values ranging from 5-48% [25] and 27-37% [27]. A higher PVC (%) indicates an efficiency of feed utilization and a better health status, which was happened in our case in V3 and V4. Similar results were reported by Aly et al., 2008, in case of dietary supplementation with different probiotics at *Oreochromis niloticus* species [28].

In variants in which were administered phytobiotics is observed a reduction of hemoglobin concentration with 13.63% in V3, 12.96% in V2 and 11.84% in V4, compared to the control variant (V1). Our results falls in the range reported by Hrubec et al., 2000, 7-9.8 g/dL [27].

Regarding, MCH and MCHC, values obtained are found in the reference range reported by Bittencourt et al., 2003 [27].

In terms of serum total protein were not registered significant differences ($p>0.05$; $p=0.25$), the values being in the reference range 4.8-7.8 g/dL [27].

At the end of the experiment for a more detailed assessment of biological material health, used in this experiment, after rosemary, sea buckthorn and ginger administration, was analyzed from serum the cortisol and lysozyme activity.

Cortisol values obtained after six weeks of the experiment are graphically presented in Figure 1.

At the end of the experiment was observed an insignificant increase ($p>0.05$; $p=0.93$) of the cortisol, in variants in which phytobiotics were administered, with 8.07% in V3, 11.07% in V2, respectively 13.82% in V4, compared with V1.

From the variants in which were administered phytobiotics, in variant with sea buckthorn was obtained the lowest value of cortisol, 396.55±123.06 ng/mL. Apines-Amar et al., 2013, reported high values of cortisol (approximately 400 ng/mL) at the *E. fuscoguttatus* species [29].

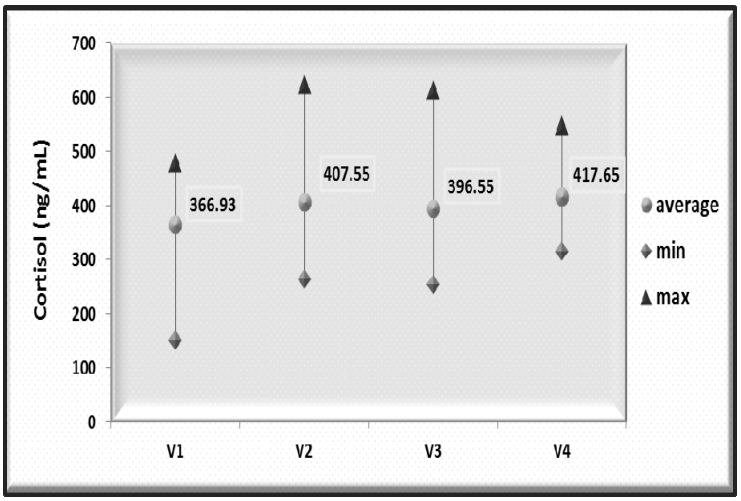

Figure 1. Variation in serum cortisol at *Oreochromis niloticus* species

Regarding lysozyme activity are observed an increased activity in V4 (9.43±0.76 U/mL), but not statistically significant compared to V1 and V2 ($p>0.05$; $p=0.30$, $p=0.49$). Ginger administration in 2% concentration at *Epinephelus fuscoguttatus* species, for eight weeks, led to an increased activity of lysozyme [29].

However, after six weeks of experiment, was observed a significant reduction ($p<0.05$; $p=0.005$) of lysozyme activity in V3. The variation of lysozyme activity is graphically presented in Figure 2.

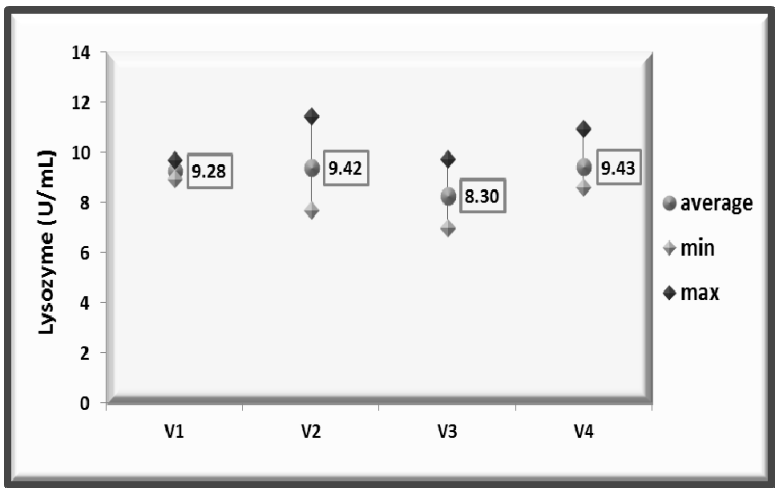

Figure 2. Variation of lysozyme activity from serum at *Oreochromis niloticus* species

For a better analysis of haematological profile was recoursed to determine the absolute number of leukocytes, respectively the achieving of leucogram (%). This is due to the fact that leukocytes play an important role in body defense at the contact with a variety of pathogens or in case of stress occurrence due to various internal or external factors.

The morphology of erythrocytes, leukocytes and thrombocytes, found on blood smears, can be seen in Figure 3.

In terms of absolute number of leukocytes were recorded significant differences between initial and final results ($p<0.05$; $p=0.04$). The highest value was recorded in V2 and the lowest in V4.

An increase in white blood cell count was registered, as well as in our case, in a hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) in which the diet was supplemented with garlic in a concentration of 0.5%, thus, leading to improvement of immunity [30]. The results of small and large number of lymphocytes showed significant differences, both, between the initial and final variants ($p<0.05$; $p=0.04$, respectively 0.007) and between final experimental variants ($p<0.05$; $p=0.049$, respectively 0.019).

Table 2. Absolute number of leukocytes and leukogram at *Oreochromis niloticus* species

Experimental variants	Vi	V1	V2	V3	V4
Absolute number (x10^3cells/µL)					
Leukocytes[a]	61.02±5.99	62.14±10.42	82.41±16.79	62.92±13.85	53.56±5.94
Small lymphocytes[ab]	59.35±5.97	60.02±10.60	80.34±16.49	61.07±13.49	51.68±6.07
Large lymphocytes[ab]	0.50±0.04	0.58±0.25	1.13±0.18	0.58±0.25	0.58±0.27
Monocytes[ab]	0.41±0.15	0.65±0.11	0.44±0.10	0.72±0.30	0.31±0.09
Neutrophils[c]	0.75±0.23	1.02±0.75	0.63±0.38	0.70±0.31	1.13±0.12
Thrombocytes[c]	21.53±3.63	20.92±10.98	25.06±5.27	25.53±5.73	33.78±9.65
Leukogram (%)					
Small lymphocytes[c]	97.19±0.68	96.43±1.49	97.47±0.66	97.03±0.63	96.40±0.70
Large lymphocytes[c]	0.84±0.09	0.78±0.37	1.21±0.46	0.67±0.21	0.88±0.52
Monocytes[ab]	0.69±0.28	1.06±0.18	0.56±0.18	1.14±0.43	0.58±0.19
Neutrophils[c]	1.28±0.44	1.73±1.4	0.76±0.37	1.16±0.63	2.14±0.24

"a"–significant differences between variants at the end of the experiment ($p<0.05$)
"b"–significant differences between initial and final experimental variants ($p<0.05$)
"c"–insignificant differences ($p>0.05$)

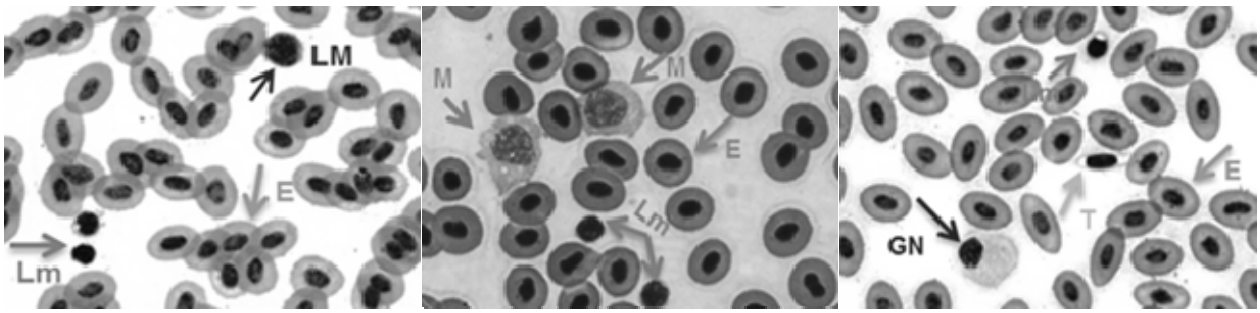

Figure 3. Morphology of cellular elements at Nile tilapia found on blood smears (photo original)
Note: E–erythrocytes; Lm–small lymphocyte; LM-large lymphocyte;
M–monocytes; GN–neutrophilic granulocyte; T–thrombocyte

The monocytes count was significantly higher in V3 compared to initial ($p<0.05$; $p=0.021$) and compared to the other experimental variants ($p<0.05$; $p=0.025$).

In terms of the neutrophils number were not registered significant differences between the experimental variants ($p>0.05$; $p=0.43$) and compared to initial moment ($p>0.05$; $p=0.48$).

The results of the thrombocytes count, at the end of the experiment, reported an increase, but not significant ($p>0.05$; $p=0.28$) in the variant in which were administered phytobiotics compared to V1. So, they increased with 19.79% in V2, 22.04% in V3 and 61.47% in V4.

Recent studies have shown that thrombocytes are involved in hemostasis process and play a defending role in organism, it is products, in teleostean fish in the spleen and kidneys [31].

This shows that administration of ginger, sea buckthorn and rosemary has improved immunity at Nile tilapia.

At blood smears analysis were not found eosinophils and basophils granulocyte.

Although, our results showed significant differences in the absolute number of leukocytes and of different types of leukocyte, the obtained values were maintained within the normal range for tilapia [25, 27].

Determination of leukogram (%) showed significant differences ($p<0.05$; $p=0.02$) between the experimental variants, only in the relative number of monocytes (%). Though, in terms of monocytes, there were significant differences in both the absolute and relative number, the maximum values were within the reference range [27].

4. Conclusions

At the end of the experiment results showed that the administration in feed of rosemary, sea buckthorn and ginger lead to significant differences ($p<0.05$) of following parameters: RBCc (x 10^6/mm^3), MCV (μm^3), glucose (mg/dl), lysozyme activity (U/mL), monocyte (%) and in absolute number of leukocytes, lymphocytes and monocytes. Although, were registered significant differences, the values obtained were within in reference intervals reported in the literature.

It was found that administration of these phytobiotics led to reduction of hemoglobin concentration compared to the control, which means an improvement of Nile tilapia physiological status. We affirm this aspect because it is very well known that hemoglobin represents an interface between the organism, environmental conditions for fish growth and technological management.

In the same time, the hematocrit percentage (PVC%) obtained in variant in which were administered ginger (30.40±3.26%) and sea buckthorn (27.80±3.49%) shows a more efficient use of feed and a better health status.

From phytobiotics administered, sea buckthorn led to lower values of glucose and plasma cortisol, indicators which stay at the base of a faster analysis of stress in fish.

Higher values of lysozyme activity obtained in V2 and V4 indicates the presence of positive effects on immunity, due to administration of ginger and rosemary.

Regarding leukogram (%), the percentage of small lymphocytes indicates immunomodulating effects of phytobiotics used, respectively immunostimulation in V2 and V3 and immunosuppression in V4.

The significant increase in the absolute number of monocytes and monocyte percentage of the leukogram level obtained in V3, may be associated with an increase in cellular defense mechanisms of the organism.

The absence of eosinophils and basophils in all experimental variants indicates an absence of inflammatory processes, which denotes a good welfare status.

In conclusion, we can say that the administration in 1% concentration of sea buckthorn and ginger, but even and rosemary administration, in diet improves the physiological status at *Oreochromis niloticus* species.

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