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SYNERGISTIC EFFECT OF *THYMUS VULGARIS* AND VITAMIN E ON HEMATOLOGICAL PROFILE, SOME BLOOD BIOCHEMICAL INDICES AND LEUKOCYTE REACTION OF *OREOCHROMIS NILOTICUS* SPECIES

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Abstract

The aim of this research was to evaluate the influence of thyme and a mixture of thyme whit vitamin E on hematological profile, some biochemical indices and leukocyte reaction of Oreochromis niloticus species, reared in a semi-intensive aquaculture system. The experiment was made in replicate, during 31 days, and the experimental variants were: V1 – 1% thyme (Thymus vulgaris)/kg feed and V2 – 1% thyme + 500 mg vitamin E/kg feed. At the end of the experiment, in V2 variant, a significant increase ($p<0.05$) were registered in case of red blood cells count (RBCc), hemoglobin concentration (Hb), mean corpuscular hemoglobin concentration (MCHC), lysozyme activity, absolute number of leukocyte (lymphocytes, monocytes) and platelets. Also, in V2 variant, was obtained a significant decrease ($p<0.05$) in case of blood glucose concentration. In conclusion, we showed that the additivited diet of Nile tilapia, particularly, with a mixture of thyme and vitamin E (V2) had a synergistic influence on fish welfare status by improving the hematological profile, immune parameters (lysozyme activity, leukocyte reaction) and decrease of blood glucose concentration.

Key words*: hematological and biochemical indices, leukocyte reaction, lysozyme activity, Nile tilapia, Thymus vulgaris.*

INTRODUCTION

In recent years researchers have approached increasingly several studies in fish hematology because it plays an important role in assessing the health status. The study of the physiological and hematological characteristics of cultured fish species is an important tool in the development of aquaculture system, particularly in regard to the use in detection of healthy from diseased or stressed animal (Rainza-Paiva et al., 2000; O'Neal and Weirich, 2001).

However, the diet composition, metabolic adaptation and variation in fish activity are the main factors responsible for the change in hematological parameters of fish (Rehulka, 2003). Some authors reported changes in blood parameters indices of fish as result of feed (Kelly, 1979; Kilgour, 1987).

The fish welfare evaluation can be done by determining the hematological profile, some blood biochemical indices and leukocyte reaction. The main haematological indices are: erythrocytes number (RBCc), hemoglobin (Hb), hematocrit (PVC) and the erythrocyte constants: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The analysis of some blood biochemical indices (total, protein, glucose, cortisol, lysozyme) and absolute and relative number of leukocytes aglucose from blood plasma is an important tool for determining the fish stress status and the immunity evaluation.

Ologhobo (1992) reported that the most common blood variables consistently influenced by diet are the RBCc, PVC, total protein and glucose levels.

Stress represents a major factor in the health of farmed fish, for this reason prevention is the ideal and it can be achieved through good management protocols, but also by development an optimal system design (Conte, 2004).
Tilapia (including all species) is the second most important group of farmed fish after carps, and the most widely grown of any farmed fish (FAO), because its ease of culture. *Oreochromis niloticus* are principally herbivorous, although occasionally they can be omnivorous. This is an efficient converter of waste foodstuff and appears to thrive well on artificial supplemental feed (Omoregie et al., 2009).
The mode of action of phytobiotics and their derivatives are attributed to the presence of many active components such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides, and flavonoids (Harikrishnan et al., 2011; Sivaram et al., 2004).
Herbs or spices have been reported to promote various functions among which hematological and biochemical status (Yılmaz and Ergun, 2012).
Among the various aromatic plants, thyme (*Thymus vulgaris* L.) is an herbaceous perennial plant belonging to the *Lamiaceae* family, and is a characteristic herb of the Tunisian and Mediterranean environment. It is recognized for its many beneficial properties: antiseptic, carminative, antimicrobial, and antioxidative properties (Baranauskiene et al., 2003). Also, thyme, has a strong antimicrobial and antioxidant activity due to its very high contents of thymol, p-cymene, carvacrol, eugenol, and 4-allylphenol (Gultepe et al., 2014). Thymol, a major component of thyme essential oils, has been widely studied for its antimicrobial properties (Dorman & Deans, 2000). Carvacrol, an isomer of thymol, is found in essential oils isolated from thyme.
Vitamins are micro-nutrients required for normal growth, reproduction, metabolism, and immune function (Safarpour et al., 2011).
Vitamin E is a fat-soluble vitamin that has two forms, tocopherols and tocotrienols. Aquatic animals cannot synthesize vitamin E, so it must be provided in their diet. Vitamin E is widely involved in anti-oxidant, cell signalling, reproductive development, immune regulation, and anti-stress processes (Galti et al., 2016; Hamre et al., 2011). It is a potent antioxidant that protects against oxidative damage in various fish tissues (Adham et al., 2000), enhances resistance of red blood cell (RBC) membranes (Kiron et al., 2004), and protects leukocyte functions (Sahoo et al., 2002).
It is an indispensable nutrient required to maintain normalphysiological functions infish (Hamre, 2011). Supplementation of dietary vitamin E has been shown to increase serum lysozyme activities in cobia (*Rachycentron canadum*) (Zhou et al., 2013) and serum complement activity in rainbow trout (*Oncorhynchus mykiss*) (Pearce et al., 2003).
Vitamin E also can regulate stress responses produced by micro cysts (Prieto et al., 2008), heavy metal pollution (Salehi et al., 2015), crowd stress (Liu et al., 2014), oxidized fish oil (Mourente et al., 2002), and high-fat diets (Lim et al., 2009). From this reason in this experiment we chose as phytobiotic the thyme and vitamin E for, well known, antioxidant effect.
The aim of this research was to investigate the influence of thyme and thyme with vitamin E combination on hematological profile and some biochemical indices of blood at *Oreochromis niloticus* species reared in a recirculating aquaculture system.

MATERIALS AND METHODS

Experimental design
This experiment was conducted in the research laboratory of the Department of Food Science, Food Engineering, Biotechnology and Aquaculture Aquaculture, from "Dunarea de Jos" University of Galati. The experimental period was 31 days. The recirculating aquaculture system includes four rearing units, with a volume of 0.5 m^3 each. For each growth units corresponded two external filters on the type Tetratec Ex 400 for water recirculation, while the water aeration was performed using a compressor and aeration stones.
In this research were used a total number of 100 Nile tilapia, with an initial average weight of 328.36 ± 37.68 g/fish, that were randomly distributed in 4 rearing units. Fish were fed with SOPROFISH pelleted feed, with 38% crude protein and 7% crude fat. The feed biochemical composition was related by Mogodan (Antache) et al. (2018). Fish were fed three times per day with a daily ration of 1%

from fish body weight. At the end of the experiment the individual average weight was 396.96 g/fish in V1 and 401.76 g/fish in V2.

The experimental variants were performed in duplicate and were organized as follows: V1 – 1% thyme (*Thymus vulgaris*)/kg feed, V2 – 1% thyme + 500 mg vitamin E/kg feed.

Blood sampling and analysis

At the beginning and at the end of the experimental period was sampling 3.5 ml of blood, from 10 fish of each growth unit, by caudal venous puncture using heparin as anticoagulant. A part of the blood was used for hematological study also, for determining glucose and total protein, and the other part was used for analysis of oxidative stress that is the subject of another study.

Blood analysis was performed by method used in fish hematology described by Blaxhall (1973). This analysis consisted in determination of erythrocytes number (RBCc x 106/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 as a diluting solution. The hematocrit was performed by duplicate using capillary tubes centrifuged 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) and Svobodova (2001) the erythrocyte constants were calculated: mean corpuscular volume (MCV, μm^3), mean corpuscular hemoglobin (MCH, pg), and 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 546 nm wavelength.

Lysozyme activity was measured, from serum, based on the turbiometric assay, Enzymatic Activity of Lysozyme Protocol (Sigma, EC 3.2.1.17). 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 450 nm 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.). The blood smears were 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). Absolute number of circulating blood leukocytes and thrombocytes was determined in comparison with 1000 erythrocytes counted on haemocytometer, per blood volume unit.

Before to start the sampling method, fish were anesthetized with 2- phenoxyethanol (8 mL/40 L of water for 5 minutes) in order to reduce handling stress. Some researchers reported that 2-phenoxyethanol anesthesia had no effect on haematological profile (Velisek et al., 2007).

Statistical analysis

The hematological parameters of the experimental groups were statistically analyzed using descriptive statistics, t - Student test (for final variants) and ANOVA single factor test (for initially moment and final variants). Programs used were Microsoft Excell 2010 and SPSS Statistics 17.0. The results were presented as mean±standard deviation.

RESULTS AND DISCUSSIONS

Svobodova et al. (1991) reported that fish hematology would be useful in the assessment of suitability of diets and feed mixtures, evaluation of fish conditions, determination of toxic effect of substances, as well as diagnosis of disease.

The red blood cell indices (PVC, Hb and RBCc) can be an indicator of oxidative status, because erythrocytes are one of the major production sites of free radical and some of them can trigger peroxidation of sutured fatty acids in their membrane phospholipids, therefore altering their quality (integrity, size) and quantity (Pearce et al., 2003; Kiron et al., 2004; Ispir et al., 2011).

The values of hematological parameters obtained at the beginning (V0) and at the end of the experiment (V1 – 1% thyme/kg feed, respectively V2 – 1% thyme + 500 mg vitamin E/kg feed), are presented in the Figures 1 - 6.

The results obtained showed significant differences ($p<0.05$) in terms of the number of erythrocytes, hemoglobin concentration, mean corpuscular hemoglobin concentration and blood plasma glucose level both between initial and final moment, as well as between final experimental variants (V1 and V2). RBC count was significantly lower ($p<0.05$) at the initial moment compared to the values obtained at the end of the experiment. Although there were significant differences ($p<0.05$) on the number of erythrocytes, the values obtained are situated in the reference interval reported by Hrubec et al. (2000) and Bittencourt et al. (2003).

At the end of the experiment the best value of hemoglobin concentration was registered in V1 variant, in which was administered thyme. Because the high value of hemoglobin content denotes the occurrence of a stressor factor we can say that the additivation of fish feed with thyme has led to stress reduction. Hemoglobins are particularly important in fish adaptation as they constitute an interface between the organism and the environment (Landini et al., 2002).

Hematocrit values increased significantly ($p<0.05$) in the experimental variants compared to the values obtained at the beginning of the experiment, but these values were situated in the reference interval indicated by Bittencourt et al. (2003).

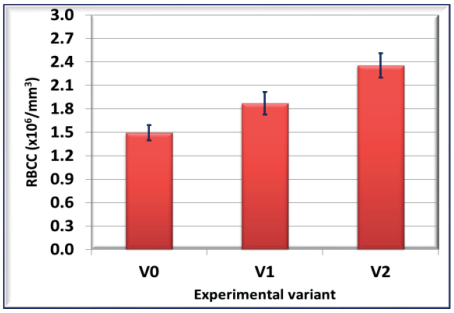

Figure 1. Changes in erythrocytes number (RBCc) of different experimental groups

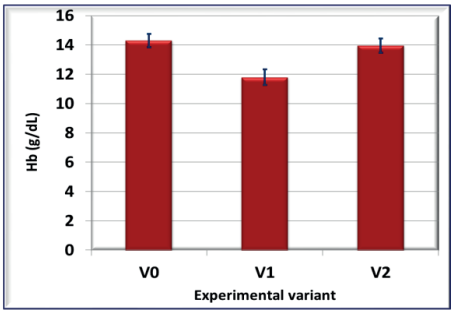

Figure 2. Changes in hemoglobine concentration (Hb) of different experimental groups

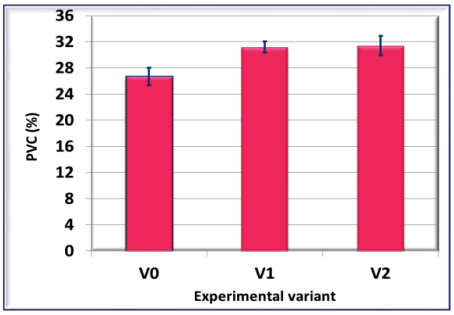

Figure 3. Changes in hematocrit (PVC) of different experimental variants during the experiment

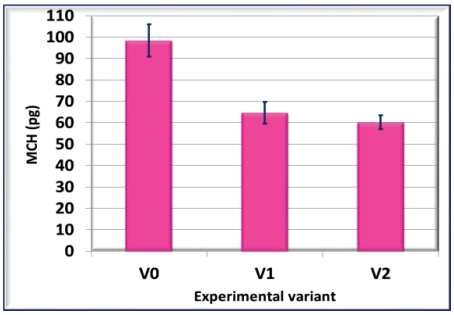

Figure 4. Changes in mean corpuscular hemoglobin (MCH) of different experimental variants during the experiment

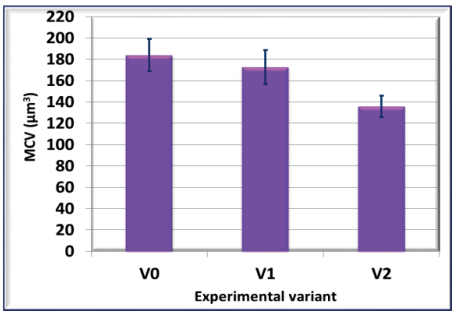

Figure 5. Changes in mean corpuscular volume (MCV) of different experimental variants during the experiment

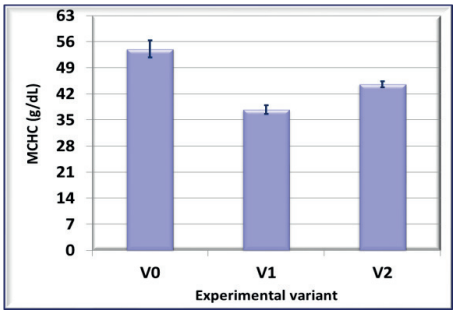

Figure 6. Changes in mean corpuscular hemoglobine concentration (MCHC) of different experimental variants during the experiment

Bai and Lee (1998) and Ispir et al. (2011) showed that the PVC of fish fed with basal diet was lower than that of fish fed with high level of vitamin E at *Sebastes schlegeli* species, respectively at *Oreochromis niloticus* species. This may explain the lower value obtained before starting the experiment. Because the increase of hematocrit was statistically significant between the initial and final moments but statistically not significant between the final variants, this shows that these hematological parameter was not relevant as an indicator of the stress response of the *Nile tilapia* induced by the introduction of thyme and thyme with vitamin E in feed.

Analyzing the results of the erythrocyte constants, it can be observed that the administration of thyme and thyme with vitamin E in feed has induced significant decrease in MCH ($p<0.05$) and MCHC ($p<0.05$) level that expresses a decrease in hemoglobin in red blood cells.

Regarding to the assessment of blood glucose levels, it is known that it is a good and fast indicator in assessing the level and intensity of stress on fish physiology. At the end of the experiment our results showed a significant decrease ($p<0.05$) in glucose level in V2 variant in which we administered thyme in combination with vitamin E in feed. We can say that the addition of vitamin E in feed, along with thyme, led to lower blood glucose level and consequently to stress reduction. However, it should be mentioned that the values obtained by us for blood glucose concentration are found in the reference interval reported by Bittencourt et al. (2003).

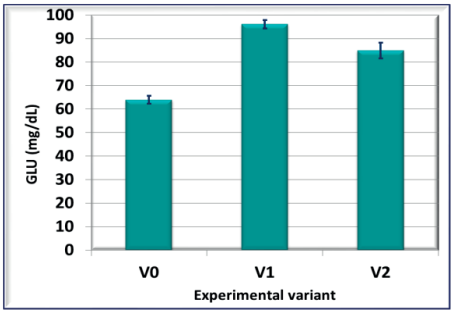

Figure 7. Changes in glucose concentration (GLU) of different experimental variants during the experiment

In case of cortisol concentration, at the end of the experiment no significant differences were obtained between the experimental variants ($p>0.05$) although, in case of glucose concentration were registered significant differences ($p<0.05$). The average value of cortisol concentration registered in variant V2 a slight increase with 9.11% compared to the value obtained in V1 variant.

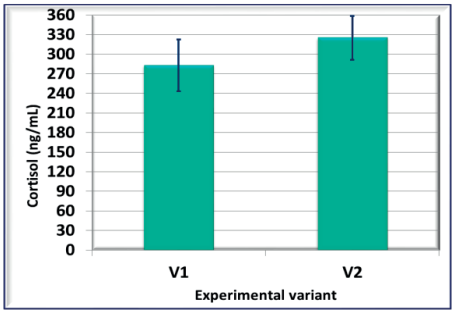

Figure 8. Changes in cortisol concentration of different experimental variants during the experiment

An increase of cortisol concentration, in the blood, represent the primary response to a stressor factor which affects the body and the

secondary response is given by blood glucose concentration (Begg and Pankhurst, 2004) we can say that the two diets administrated to *Nile tilapia* did not influence the occurrence of stress. So, either diet can improve the physiological state of the biological material.
From this reason, for a better assessment of the biomass culture in terms of occurrence of stress should not only determine the concentration of glucose in the blood because if an experiment glucose concentration may indicate certain differences between experimental variants.
Regarding the total protein from blood serum, the results showed insignificant differences both between the initial and final moment of the experiment ($p>0.05$) as well as between the final experimental variants, V1 and V2 ($p>0.05$). The values obtained are included in the reference interval indicated by Bittencourt et al. (2003). It should be noted that the measurement of total protein, from serum or plasma, is considered a diagnostic value in fish because it relates to general fish nutritional status, as well as to the integrity of the vascular system and liver functions (Abdel-Tawwab et al., 2008).

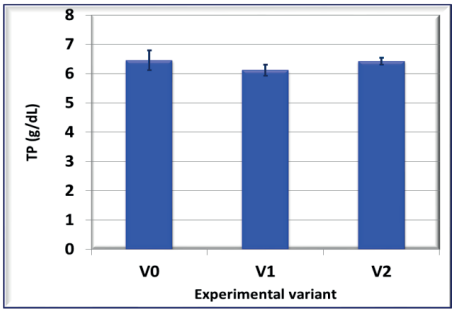

Figure 9. Changes in total protein concentration (TP) of different experimental variants during the experiment

Determination of lysozyme activity was performed in order to observe the resistance capacity of the fish biomass in case of disease. Moreover, lysozyme is a bacteriolytic enzyme that plays an important role in the defence of the body, because it has antibacterial and immuno-modulating properties (Takahashi and Itoh, 2011).
At the end of the experiment, the results of lysozyme activity showed a significant differences ($p<0.05$) between experimental variant. In variant V2, in which fed was additivated with thyme and vitamin E, was registered an intensification of the lysozyme activity.

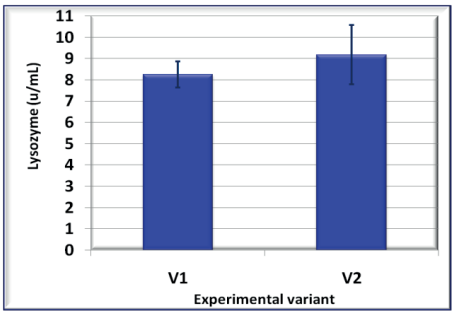

Figure 10. Changes in lysozyme activity (Lys) of different experimental variants during the experiment

For a better assessment of the health status of the biological material cultured under the conditions of the administration of different diets, the reactions of the leukocytar system was also analyzed. This analysis involved the determination of the relative and absolute number of leucocytes that can give us clear information about the possible changes that have occurred at the physiological level and the state of health in which the fish are.
After analysing the blood smears the leukogram of the studied specimens revealed the presence of agranulocytes, including small lymphocytes (Lm), large lymphocytes (LM), and the presence of neutrophilic granulocytes (N). Basophilic and eusinophilic granulocytes were absent. In figures 11, 12 and 13 are presented the leukograms (%) obtained from the blood smear analysis at the beginning and at the end of the experiment.
At the end of the experiment, the analysis of the relative number of leukocytes highlighted the aspects detailed below.
Lymphocytes (%). Regarding the small lymphocytes (Lm) between the experimental variants, there were no significant differences ($p>0.05$), but the highest value was recorded in variant V2 (97.35%). Although the initial value of the relative number of small lymphocytes was higher than the values obtained at the end of the experiment, no significant differences were obtained ($p>0.05$) (Figures 11, 12, 13). The relative number of large lymphocytes (LM) registered insignificant differences between the final experimental variants

(p>0.05) and neither between the initial mean value and the mean values obtained at the end of the experiment (p>0.05). However, compared to the initial value there was an increase of 82.61% in V1 and 86.96% in V2.

Monocytes (%). At the end of the experiment was found an increase of monocytes (M, %) in the experimental variants, compared to the average value obtained at the beginning of the experiment (0.33%), but insignificant (p>0.05) from statistical point of view Figures 11, 12, 13. In variant V2, in which thyme was administered in combination with vitamin E, was observed a decrease with 16.8% of the relative number of monocytes (%), compared to variant V1, but also insignificant (p>0.05).

Neutrophilic granulocytes (%). If at the level of the relative number of lymphocytes (%) and monocytes (%) there were not registered significant differences (p>0.05), regarding the relative number of neutrophils (%) a significant increase was obtained in V1 variant (1.99%), compared to V2 variant (p<0.05) and initial (V0) value (p<0.05).

The morphology of erythrocytes, leukocytes and thrombocytes, found on blood smears, can be seen in Figure 14.

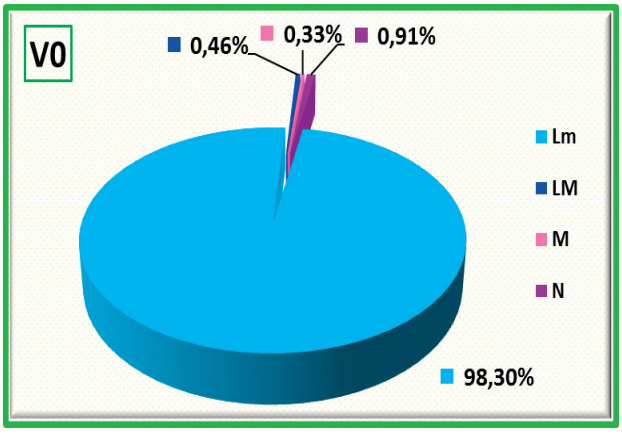

Figure 11. Nil tilapia leukogram in V0 variant at the beginning of the experiment

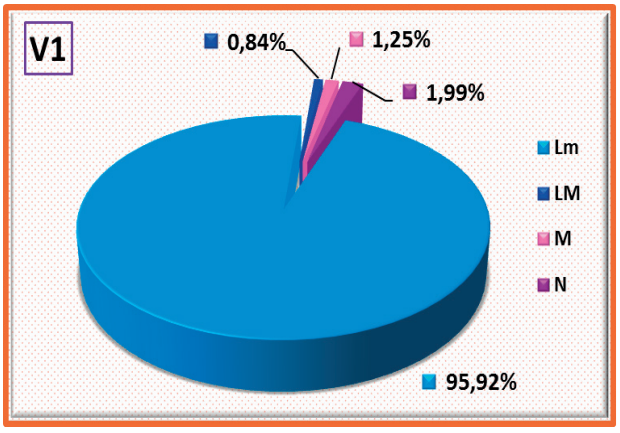

Figure 12. Nil tilapia leukogram in V1 variant at the end of the experiment

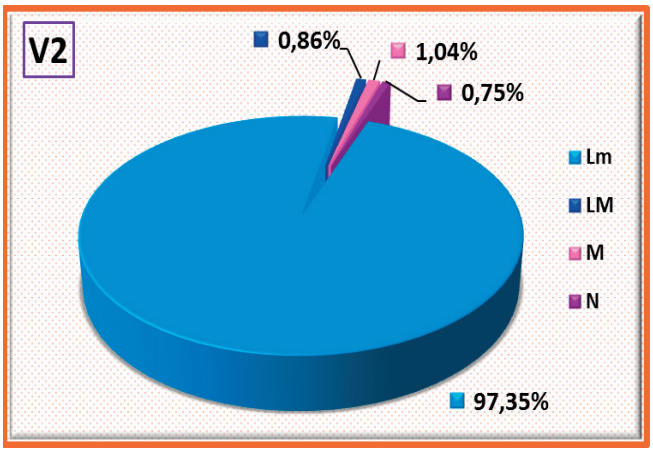

Figure 13. Nil tilapia leukogram in V2 variant at the end of the experiment

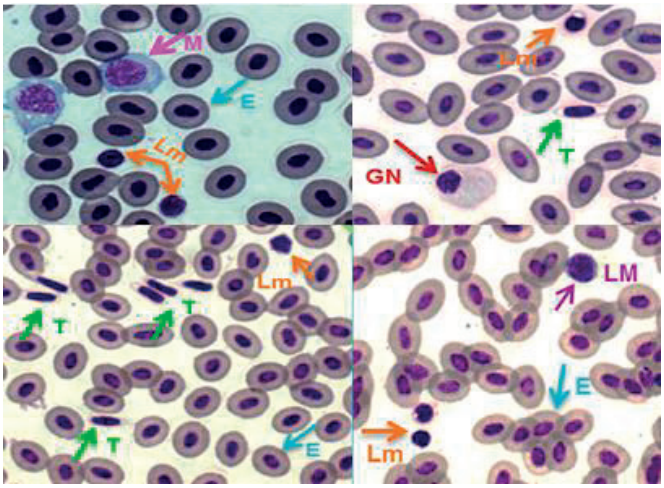

Figure 14. Morphology of cellular elements at Nile tilapia found on blood smears (photo original)
Note: E - erythrocytes; Lm - small lymphocyte; LM - large lymphocyte; M - monocytes; GN - neutrophilic granulocyte; T - thrombocyte

In order to obtain a clearer picture of the changes that take place in the leukocyte array, besides determining the relative number (%), the absolute number of white blood cells and the absolute number of platelets (number of cells/mm^3 blood) were calculated. The absolute modifications of the various types of cells that form the leukocytic complex are presented in Table 1.

Table 1. Variation in the absolute number of leukocytes and platelets at *Nile tilapia* during the experiment

Var. exp.	Absolut number (x1000 cel./mm^3)					
	Leukocytes	Lymphocytes		Monocytes	Neutrophilic granulocytes	Platelets
		small	large			
V0	56.83±62.68	55.86±6.54	0.26±0,06	0.18±0.11	0.53±0.11	4.10±1.41
V1	69.37±14.73	66.68±14.78	0.67±0,40	0.81±0.09	1.33±0.44	32.89±18.15
V2	114.91±39.45	112.18±39.07	1.12±0.72	1.07±0.52	0.78±0.27	45.47±8.57

Note: Exp. Var. – experimental variant. Results are expressed as mean±standard deviation.

Absolute number of leukocytes. At the end of the experiment, there was a significant increase ($p<0.05$) of the leukocytes number in the V2 variant compared to the initial average value, respectively with 102.20%. There were no significant differences between variants V1 and V2 ($p>0.05$), although the value obtained in V1 was with 39.63% lower compared to the mean value obtained in V2 (Table 1). Our values are registered within the reference range 21559-154690 leukocytes/mm^3 (Hrubec et al., 2000).

Absolute number of small lymphocytes. Because the small lymphocytes represent the largest proportion of the total leukocytes, also in their case were registered significant differences between the number obtained in variant V2 and the number of small lymphocytes obtained at the beginning of the experiment ($p<0.05$). It increased with 100.82% in V2, respectively with 19.37% in V1, compared to the initial absolute number of small lymphocytes. During the experiment the average values of the number of small lymphocytes were between 55.86 x 10^3 cel/mm^3 and 112.18 x 10^3 cell/mm^3. These are in the range presented in the literature for tilapia (6.78-136.39 x 10^3 cel/mm^3) (Hrubec et al., 2000).

The ***absolute number of large lymphocytes*** shows the same growth tendency at the end of the experiment as the number of small leukocytes and lymphocytes, but they were not registered significant differences ($p>0.05$). The number of large lymphocytes increased with 157.69% in V1, respectively with 330.77% in V2, compared to the initial value (0.26 x10^3 cel/mm^3) (Table 1). The number of large lymphocytes recorded was lower than that recorded by Hrubec et al. (2000) (2.85-30.83 x10^3 cel/mm^3).

Regarding to the ***absolute number of monocytes***, were registered significant differences between the values obtained at the end of the experiment compared to the initial (V0) value ($p<0.05$). The highest number of monocytes was recorded in the variant in which thyme was administered in combination with vitamin E (V2) - 1.07 x 10^3 cel/mm^3, and the lowest in the variant in which thyme was administered (V1) - 0.81 x 10^3 cel/mm^3 (Table 1). At the end of the experiment were not recorded significant differences between the experimental variants ($p>0.05$). If the absolute number of leukocytes obtained at the end of the experiment falls within the reference range; 0.40-4.29x10^3 cel/mm^3 (Hrubec et al., 2000); the number of monocytes obtained at the beginning of the experiment does not fall within the range, being smaller - 0.18x10^3 cel/mm^3.

The ***absolute number of neutrophilic granulocytes*** registered a significant increase in case of V1 variant was administered, compared to the variant V2 ($p<0.05$) and compared to the absolute number determined at the beginning of the experiment ($p<0.05$). The number of neutrophils increased from the initial moment with 150.94% in the V1 variant, respectively with 47.17% in the V2 variant (Table 1). The mean values of the absolute number of neutrophil granulocytes ranged between 0.53±0.11 x 10^3cel/mm^3 and 1.33±0.44 x 10^3 cel/mm^3 (Table 1). The results obtained are found in the optimum range for tilapia; 0.56-9.87 x 10^3 cel/mm^3 (Hrubec et al., 2000).

Neutrophilic granulocytes represent the first white blood cells who arrive in the place where the tissue is lysed due to a particular pathogen (in case of infections) (Toazza et al., 2013). These have a very important role in ensuring native immunity because they have the ability to recognize, phagocyte and destroy the pathogen without stimulating other cells that play a role in the defense of the body (Tavares-Dias and Moraes, 2007). Although, the number of neutrophils increased significantly at the end of the experiment, it can not be about the presence of an infection because the obtained results fall within the optimal limits for tilapia.

The ***absolute number of platelets*** registered a significant difference ($p<0.05$) between the average value obtained at the beginning of the experiment (4.10±1.41 x 10^3 cel/mm^3) and the average values obtained at the end of the experiment in V1 variant (32.89±18.15 x 10^3 cel/mm^3) and V2 variant (45.4±8.57 x 10^3 cel/mm^3). In V2 variant, the absolute number of platelets increased with 38.25% compared to V1 variant, but this was insignificant ($p>0.05$). In tilapia, the optimal range for absolute platelet count ranges from 25.06 x 10^3 cel/mm^3 to 85.24 x 10^3 cel/mm^3 (Hrubec et al., 2000). Thus, the results showed that the number of platelets from the beginning of the experiment

is below than lower limit described by Hrubec et al. (2000), but according to Tavares-Dias and Oliveira (2009), who claim that in fish the number of platelets is between 2.00-78.90 x10^3 cel/mm^3, the results obtained being less than inferior limit.
The low number of platelets from the beginning of the experiment showed that the fish have a deficient immune system, being more sensitive to the attack of different pathogens (Tavares-Dias and Oliveira, 2009). This is also certified by the small number of leukocytes, more specifically lymphocytes, recorded at the beginning of the experiment. Ndong and Fall (2011) showed that the leukocytes have grown in the variant in which they administered garlic, to a tilapia hybrid, in 0.5% concentration, fish immunity was also improved.
In fish, increasing the leukocyte numbers in circulating blood may be associated with increasing of leukocyte production in the hematopoietic tissue of the kidney, respectively in the spleen (Manolescu, 1999). It has been shown that the main function of lymphocytes is to produce antibodies and other chemicals that serve to protect the body against infection (Ramesh & Saravanan, 2008). Thus, it is confirmed that the administration of thyme in combination with vitamin E has led to a significant improvement of immunity due to increase in number of lymphocytes. The administration of thyme (V1) has contributed to the improvement of the immune system in a smaller proportion.

CONCLUSIONS

In the past few years for ensuring the fish welfare status will be introduced phytobiotics in fish feed against antibiotics and other medicinal products administration. The main disadvantages of using different types of medicines are represented by high costs and environmental pollution.
This experiment reveals that the thyme in combination with vitamin E shows a synergistic effect leading to a decrease of blood glucose level, but, due to the decrease of RBCc, Hb and MCHC in V1, we can say that the introduction of thyme in Nile tilapia diet not constitute a stressor factor from the physiological point of view.
Also, the increase in leukocyte numbers, especially in lymphocytes and monocytes, respectively in the number of platelets, showed an improvement of the fish immunity in the variant in which fisf fed was additivated with thyme and vitamin E (V2).
At the same time, the blood biochemical analysis reflects a better condition in the variant in which thyme was administered in combination with vitamin E, due to the results obtained in case of cortisol, total proteins and lysozyme activity.
In conclusion, in our case, the additivited diet of Nile tilapia, particularly, with a mixture of thyme and vitamin E (V2) had a synergistic influence on the fish welfare status by improving the hematological profile and immune parameters.

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REFERENCES

Abdel-Tawwab, M., Abdel-Rahman, A. M., Ismael, N. E. M. (2008). Evaluation of commercial live bakers' yeast, Saccharomyces cerevisiae as a growth and immunity promoter for fry Nile tilapia, *Oreochromis niloticus* (L.) challenged in situ with *Aeromonas hydrophila*. *Aquaculture,* 280, 185–189.

Adham, K. G., Hashem, H. O., Abu-Shabana, M. B., Kame, A., H. (2000). Vitamin C deficiency in the catfish (Clarias gariepinus), *Aquacult. Nutr*., 6, 129–139.

Bai, S. C., Lee, K. J. (1998). Different levels of dietary DL-alpha-tocopheryl acetate affect the vitamin E status of juvenile Korean rock fish, *Sebastes schlegeli*. *Aquaculture*, 161, 405–414.

Baranauskiene, R., Venskutonis, P. R., Viskelis, P., Dambrauskiene, E. (2003). Influence of nitrogen fertilizers on the yield and composition of thyme (*Thymus vulgaris*). *Journal of Agricultural and Food Chemistry*, 51, 7751–7758.

Begg, K., Pankhurst, N.W., (2004). Endocrine and metabolic responses to stress in a laboratory population of the tropical damselfish Acanthochromis polyacanthus. *Journal of Fish Biology*, 64(1), 133–145.

Bittencourt, N.L.R., Molinari, L.M., Scoaris, D.O., Pedroso, R.B., Nakamura, C.V., Nakamura, T.U.,

Abreu Filho, B.A., Dias Filho, B.P. (2003). Haematological and biochemical values for Nile tilapia *Oreochromis niloticus* cultured in semi-intensive system. *Acta Scientiarum. Biological Sciences Maringá*, 25(2), 385–389.

Blaxhall, P. C., Daisley, K. W. (1973). Routine Haematological Methods for Use Fish Blood. *Journal of Fish Biology*, 5 (6), 771–781.

Conte, F. S. (2004). Stress and the welfare of cultured fish. *Appl. Anim. Behav. Sci.* 86, 205–223.

Dorman, H. J. D., Deans, S. G. (2000). Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, 88, 308–316.

Galli, F., Azzi, A., Birringer, M., Cook-Millsd, J. M., Eggersdorfere, M., Frankf, J. (2016). VitaminE:emerging aspects and new directions. *Free Radical Biol. Med.,* 10216–36.

Ghergariu, S., Pop, A., Kadar, L., (1985), *Veterinary Clinical Laboratory Guide*, Bucharest.

Gültepe, N., Bilen, S., Yılmaz, S., Güroy, D., and Aydın, S. (2014). Effects of herbs and spice on health status of tilapia (*Oreochromis mossambicus*) challenged with *Streptococcus iniae*. *Journal Acta Veterinaria Brno*, 83, 125–131.

Hamre, K., (2011). Metabolism, interactions, requirements and functions of vitamin E in fish, *Aquacult. Nutr.* 17, 98–115.

Harikrishnan, R., Balasundaram, C., Heo, M.-S. (2011). Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish, Aquaculture 317, 1–15.

Hrubec, T.C., Cardinale, J.L., Smith, S.A., (2000). Hematology and Plasma Chemistry Reference Intervals for Cultured Tilapia (*Oreochromis Hybrid*), *Veterinary Clinical Pathology*, 29(1), 7–12.

Ispir, U., Yonar, M. E., Oz, O. B. (2011). Effect of dietary vitamin E supplementation on the blood parameters of Nile tilapia (*Oreochromis niloticus*). *The Journal of Animal & Plant Sciences*, 21 (3), 566–569.

Kelly, W. R. (1979). *Vet. Clinical Diagnosis (2nd edition) Balliere* Tindall, London.

Kilgour, O. F. G. (1987). *Mastering Nutrition, Macmillan Education Limited*, London.

Kiron, V., Puangkaew, J., Ishizaka, K., Satoh, S., Watanabe, T., (2004). Antioxidant status and nonspecific immune responses in rainbow trout (*Oncorhynchus mykiss*) fed two levels of vitamin E along with three lipid sources. *Aquaculture* 234, 361–379.

Landini, G. F., Schwantes, A. R., Schwantes, M. L. (2002). *Astyanax scabripinnis* (Pisces: Characidae) hemoglobins: structure and function. *Braz J. Biol*, 6, 595–599.

Lim, C., Yildirim-Aksoy, M., Li, M. H., Welker, T. L., Klesius, P. H. (2009). Influence of dietary levels of lipid and vitamin E on growth and resistance of Nile tilapia to *Streptococcus iniae* challenge. *Aquaculture* 298, 76–82.

Liu, B., Xu, P., Xie, J., Ge, X. P., Xia, S. L., Song, C.Y. (2014). Effect of emodin and vitamin E on the growth and crowding stress of Wuchang Bream (*Megalobrama amblycephala*), *Fish Shellfish Immunol*. 40, 595–602.

Manolescu, N. (1999). *Tratat de hematologie animală Vol. I*, Editura Fundatiei Romania de maine, Bucuresti.

Mogodan (Antache), A., Docan, A., Dediu, L., Grecu, I., Cristea, V., Petrea, S. M., Cretu, M. (2018). Effects of some phytobiotics on oxidative stress in *Oreochromis niloticus* reared in a recirculating aquaculture system. *AACL Bioflux*, 11(1), 211-220.

Mourente, G., Diaz-Salvago, E., Bell, J. G. (2002) Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead seabream (*Sparus aurata*) fed dietary oxidized oil: attenuation by dietary vitamin E. *Aquaculture,* 214, 343–361.

Ndong, D., Fall, J., (2011). The effect of garlic (*Allium sativum*) on growth and immune responses of hybrid tilapia (*Oreochromis niloticusxOreochromis aureus*), *Journal of Clinical immunology and Immunopathology Research*, 3(1), p. 1-9.

Ologhobo, A. D., (1992). Nutritive Values of Some Tropical (West African) Legumes for Poultry. *Journal Appl. Animal Res*., 2, 93 – 104.

Omoregie, E., Igoche, L., Ojobe, T.O., Absalom, K.V., Onusiriuka, B.C. (2009). Effect of varying levels of sweet potato (*Ipomea batatas*) peels On Growth, feed utilization and some biochemical responses of the cichlid (*Oreochromis niloticus*). *African Journal of Food Agriculture Nutrition and Development*, 9(2), 700–712.

O'Neal, C.C., Weirich, C.R. (2001). Effects of low level salinity on prod. and haematological parameters of channel catfish, *Ictalurus punctatus* reared in multicrop ponds. In: Book of abstract. *Aquaculture* 2001. Int. Triennal Conf. of World Aquaculture Soc. Jan. 21-25, 2001. Disney Colorado Springs Resort Lake Buena Vista, Florida, p. 484.

Pearce, J., Harris, J. E., Davies, S. J. (2003). The effect of vitamin E on the serum complement activity of the rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquacul Nut.*, 9, 337–340.

Prieto, A. I., Jos, Á., Pichardo, S., Moreno, I., Cameán, A. M. (2008), Protective role of vitamin E on the microcys induced oxidative stress in tilapia fish (*Oreochromis niloticus*). *Environ. Toxicol. Chem.,* 27, 1152–1159.

Rainza-Paiva, M.J.T., Ishikawa, C.M., Das Eiras, A.A., Felizardo, N.N. (2000). *Haemotological analysis of Chara, Pseudoplatystoma fasciatum in captivity. Aqua 2000. Responsible aquaculture in the new millennium*. May 2-6 2000. European Aquaculture Soc. Special Pub. Nice, France, p. 590.

Ramesh, M., Saravanan, M., (2008). Haematological and biochemical responses in a freshwater fish *Cyprinus carpio* exposed to chorpyrifos. *Int J Integrative Bio*, 3(1), 80–83.

Rehulka, J. (2003). Haematological and biochemical analysis in rainbow trout, *Oncorhynchus mykiss* affected by Viral Haemerhagic Septicaemia (VHS). *Dis. Aquat. Org.*, 56, 186–193.

Safarpour, A., Falahatkar, B., Sattari, M., Tolouei Gilani, M. H. (2011). Effect of dietary vitamin E on growth, muscle composition, hematological and

immunological parameters of sub-yearling beluga *Huso huso* L. Fish Shellfish Immunol., 30, 807–814.

Sahoo, P. K., Mukherjee, S. C. (2002). Influence of fish dietary α-tocopherol intakes on specific immune response, non-specific immune resistance factors and disease resistance of healthy and aflatoxin B1 induced immune-compromised Indian major carp, Labeorohita (Hamilton). *Aquacult, Nutr.,* 8, 159–167.

Salehi, I., Karamian, R., Komaki, A., Tahmasebi, L., Taheri, M., (2015). Efects of vitamin E on lead-induced impairments in hippocampal synaptic plasticity. *Brain Res.,* 1629, 270–281.

Sivaram, V., Babu, M., Immanuel, G., Murugadass, S., Citarasu, T., Marian, M. (2004). Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against *Vibrio harveyi* infections. *Aquaculture*, 237, 9–20.

Svobodova, Z., Fravda, D., Palakova, J. (1991), *Unified methods of haematological examination of fi*sh. Research Institute of fish Culture and Hydrobiology, VURH Vodnany, Edice Metodik, Czechoslovakia,.

Svobodova, Z. (2001). Stress in Fishes (A Review). *Bull. Vurh Vodnany*, 4, 169–191.

Takahashi, K.G., Itoh, N., (2011). *Lysozymes in molluscs*, 93-102. In Bondad-Reantaso, M.G., Jones, J.B., Corsin, F., Aoki, T. (eds.). Diseases in Asian Aquaculture VII, Fish Health Section, Asian Fisheries Society, Selangor, Malaysia, p. 385.

Tavares-Dias, M., Oliveira, S. R., (2009). A review of the blood coagulation. *Revista Brasileira de Biociências, Porto Alegre*, 2009, 7(2), 205–224.

Tavares-Dias, M., Moraes, F.R., (2007). Leukocyte and thrombocyte reference values for channel catfish (*Ictalurus punctatus* Raf.), with an assessment of morphological, cytochemical, and ultrastructural features. *Veterinary Clinical Pathology*, 36, 49–54.

Toazza, C., Carandina, L., Salvador, R., Loyola,W., Freitas, J.C., (2013). ArtinM as a Neutrophil Immunostimulant in Juvenile Nile Tilapia (Oreochromis niloticus). *Open Journal of Veterinary Medicine*, 3, 204–208.

Velíšek, J., Svobodová, Z., and Piačková, V., (2007). Effects of 2-Phenoxyethanol Anaesthesia on Haematological Profile on Common Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncorhynchus mykiss*), *Acta Vet. Brno*, 76, 487–492.

Yılmaz, S., Ergün, S. (2012). Effects of garlic and ginger oils on hematological and biochemical parameters of sea bass, *Dicentrarchus labrax*. *J. Aquat. Anim. Health*, 24 (4), 219–224.

Q.C. Zhou, L.G. Wang, H.L. Wang, T. Wang, C.Z. Elmada, F.J. Xie, (2013). Dietary vitamin E could improve growth performance, lipid peroxidation and non-specific immune responses for juvenile cobia (*Rachycentron canadum*), *Aquacult. Nutr.*, 19, 421–429.

http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en#tcNA00D9

Enzymatic Activity of Lysozyme Protocol – Sigma Aldrich. Home page address: http://www.sigmaaldrich.com/technicaldocuments/protocols/biology/enzymatic-assay-oflysozyme.html