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 **ORIGINAL PAPEER**

# Virulence, pathogenicity, antibiotic resistance and plasmid profile of *Escherichia coli* strains isolated from drinking waters

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## Abstract
The aim of this study was to investigate the antibioresistance profile and the virulence and pathogenicity hallmarks of *Escherichia coli* acquatic strains. Material and methods. 50 environmental *Escherichia coli* were isolated from drinking water in Constanta, Romania. The disc diffusion susceptibility test was used to investigate the antibiotic resistance profile of these bacteria. The rapid test to nitrocephine was used for the confirmation of the presence of β-lactamases. The analysis of plasmidial DNA was performed using Wizard extraction kit. The virulence tested features were: adherence capacity on HeLa cells by Cravioto adapted method, adherence on inert substrata quantified by slime test, production of extracellular enzymes and exotoxins (haemolysins and other pore-forming toxins, amylase, mucinase, gelatinase, caseinase, aesculin hydrolysis). Results and discussions. The results of the present study have shown that aquatic medium signifies an appropriate ecological system for the existence and maintenance of a complex reservoir of antibioresistance and virulence factors with high risk for human host colonization and implications in the human health.

**Keywords**: *Escherichia coli*, virulence, pathogenicity, antibiotic resistance.

## Introduction
Contamination of surface waters by fecal pollution raises a serious environmental and public health threat. In large complex systems, fecal pollution can be introduced from multiple sources, including sewage overflows, agricultural runoff, and urban stormwater. Identifying and eliminating the source of contamination is not straightforward because assessment of fecal pollution generally relies on a limited number of surface water samples to measure fecal indicator organism densities (Byappanahalli et al., 2003; Gordon et al., 2002). *E. coli* is a type of fecal coliform bacteria commonly found in the intestines of

animals and humans. The presence of *E. coli* in water is a strong indication of recent sewage or animal waste contamination. During rainfalls, snow melts, or other types of precipitation, *E. coli* may be washed into creeks, rivers, streams, lakes, or ground water. When these waters are used as sources of drinking water and the water is not treated or inadequately treated, *E. coli* may end up in drinking water (Llopis et al., 2004). Numerous studies provide evidence that *E. coli* can persist in the benthos environment and subsequently be detected in overlying surface waters (Torrella et al., 2003). Residual populations were reported in one study, where fecal coliform levels in wastewater subjected to low temperatures decrease rapidly but then stabilize to 1 - 10% of the initial population size. In addition, *E. coli* that has been isolated from septic tanks has been found to be less diverse and genetically distinct than strains of *E. coli* from the inhabitants of the households served by those systems. Although most *E. coli* strains are harmless and live in the intestines of healthy humans and animals, this strain could exhibit powerful virulence factors and can cause severe illness with a large spectrum of etiologies. The aim of the present study was to investigate the antibioresistance profile, the virulence and pathogenicity hallmarks of *E. coli* acquatic strains.

**Material and methods**

Bacterial strains. In this study there were analyzed 50 environmental *E. coli* strains isolated in Constanta, Romania, from drinking water. The isolation and identification of these strains is based on filter membrane method, according to SR ISO 9308-1 2000. This technique consists in filtering 100 ml water sample using a filter membrane of 47mm diameter. The membrane is applied on Lactose TTC medium poured in 47 mm diameter Petri plates. After 48 hours incubation at 37°C, *E. coli* will develop yellow colonies on the membrane. Oxidase and indole production test were performed additionally for the identification of *E. coli* strains.

Antibiotic susceptibility profiles of the strains were determined by the disc diffusion method. Plates of Mueller-Hinton agar were inoculated with a bacterial suspension equivalent to a 0.5 McFarland standard and incubated aerobically at 37°C for 18 h. Results were expressed as susceptible or resistant according to the criteria adopted by the NCCLS (2006), using the following antibiotics: cefoxitin (FOX), cefotaxime (CTX), amoxicillin/clavulanic acid (AMC), imipenem (IMP), cefuroxime (CXM), tetracycline (TET), doxycycline (DOX), ampicillin (AM), ticarcillin (TIC), tobramycin (NN), gentamycin (GN), amikacin (AN), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), pefloxacine (PEF), nalidixic acid (NA), (Oxoid, Basingstoke, Hampshire, England disks).

Confirmation of β-lactamases production was performed by nitrocephine chromogenic test, double disk diffusion test (Maluping et al., 2004; Severo et al., 2002) and MICs for ß-lactams determined by using nutrient broth microdillution

method and E-test ESBL strips (AB Biodisk, Dalvägen, Sweden) (Maluping et al., 2004; Severo et al., 2002).

The molecular approach of the antibioresistance was performed by the analysis of plasmidial DNA (performed using Wizard extraction kit, Promega) (Maluping et al., 2004; Pavlov et al., 2004; Severo et al., 2002).

Cell-associated virulence factors assay

The bacterial ability to colonize the abiotic surface was quantified by slime test (Christensen et al., 1982). The strains were cultivated in tubes with nutrient broth and incubated at 37°C for 24 h and thereafter the cultures tubes were emptied and stained with safranin alcoholic solution 1% for 30 minutes, washed three times with distilled water and left at room temperature for 24 h. The intensity of the red ring on the tube glass wall was noted with +, ++, +++, ++++.

The adherence capacity to the biotic substrate (HeLa cells) was investigated by using Cravioto method (adapted by us) (Lazăr, 2003). In this purpose 1 ml bacterial suspension prepared from a broth culture of 24 h was inoculated on (80%) confluent cellular layer of HeLa-2 cells. After an incubation of 2 h at 37°C, the bacterial suspension was discarded and the cell culture washed and colored by Giemsa method. The adhesion was microscopically examined for the identification of the adhesion patterns (i.e. diffuse, localized and aggregative) and for the quantification (+, ++, +++, ++++) points of view.

Soluble enzymatic factors implicated in bacterial virulence

The CAMP-like factor was evidenced by streaking the tested strains at 8 mm distance from the β-haemolysis producing Staphylococcus aureus (ATTC 25923) and Rodococcus equi (ATCC 6939) on 5% sheep blood agar plates and incubated aerobically at 37°C for 24 h. The synergistic clear haemolysis noticed at the junction of the two spots areas, often with an arrow-like appearance, indicated the production of CAMP-like factor. The plate hemolysis was evidenced by streaking the tested strains on blood agar plates containing 5% (vol/vol) sheep blood in order to obtain isolated colonies. After incubation at 37°C for 24 h, the clear areas (total lysis of red blood cells) around the colonies were registered as positive reactions. For the investigation of lipase production the strains were spotted on Tween 80 agar as a substrate at a final concentration of 1 % and were incubated at 37°C until 7 days. An opaque (precipitation) zone around the spot was registered as positive reaction. For lecithinase production, the cultures were spotted into 2.5% yolk agar and incubated at 37°C for 7 days. An opaque (precipitation) zone around the spot indicated the lecithinase production. Decarboxilases: LDC are active (efficient) in anaerobiose, at acid pH and catalyzes in presence of phosphate pyridoxal coenzyme, the ornitine (dyamino-monocarboxilic aminoacids) and lysine-decarboxilation reaction involves the specific dyamines appearance. In practice, the ODC, LDC (lysindecarboxilase) and ADH detection is made by evidencing the pH medium variation. The glucose fermentation by bacteria with

fermentative metabolism requires culture medium acidity and the medium color turns in to yellow. If the bacteria don't express lysindecarboxilase in their enzymatic equipment, the medium remains yellow and, in presence of these enzymes, there it will be a secondary realkalinisation of the medium due to dyamine-cadaverine production and the medium color turns again into purple. The DN-ase production was studied on DNA supplemented medium. The strains were spotted and after incubation at 37°C for 24 h, a drop of HCl 1N solution was added upon the spotted cultures; a clearing zone around the culture was interpreted as positive reaction. The caseinase activity was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37°C for 24 h, a clearing zone surrounding the growth indicated casein proteolysis. The mucinase production was determined using pig stomach mucine (final concentration of 1%) incorporated in brain heart agar with 2% NaCl. The strains were spotted and incubated at 35°C for 48 h; the enzyme activity was noticed by the presence of a clear area around the culture spot; the clear area became more evident when some Lugol drops were poured upon. The amylase production was tested on agar medium supplemented with 10% starch. The strains were stubbed and incubated at 37°C for 24 h, starch hydrolysis was registered by the presence of a clear area around the culture spot (Centre de l'Enseignement de l'Institut Pasteur de Paris., 2000; Figura and Guglielmetti, 1987 Lenette et al., 1980; Wiggins et al., 2001.).

## Results and Discussion

**Antibiotic susceptibility data**. The aquatic *E. coli* strains were generally susceptible to the majority of tested antibiotics. Irrespective to the source of isolation the tested strains exhibited resistance to doxycycline, tetracycline, ampicillin and ticarcillin (fig. 1). The tested strains exhibited between 1 and 5 antibioresistance markers, the most frequent associations being: AM + TET (12%); AM + DOX (10%) and AM + TIC (8%) (fig. 2).

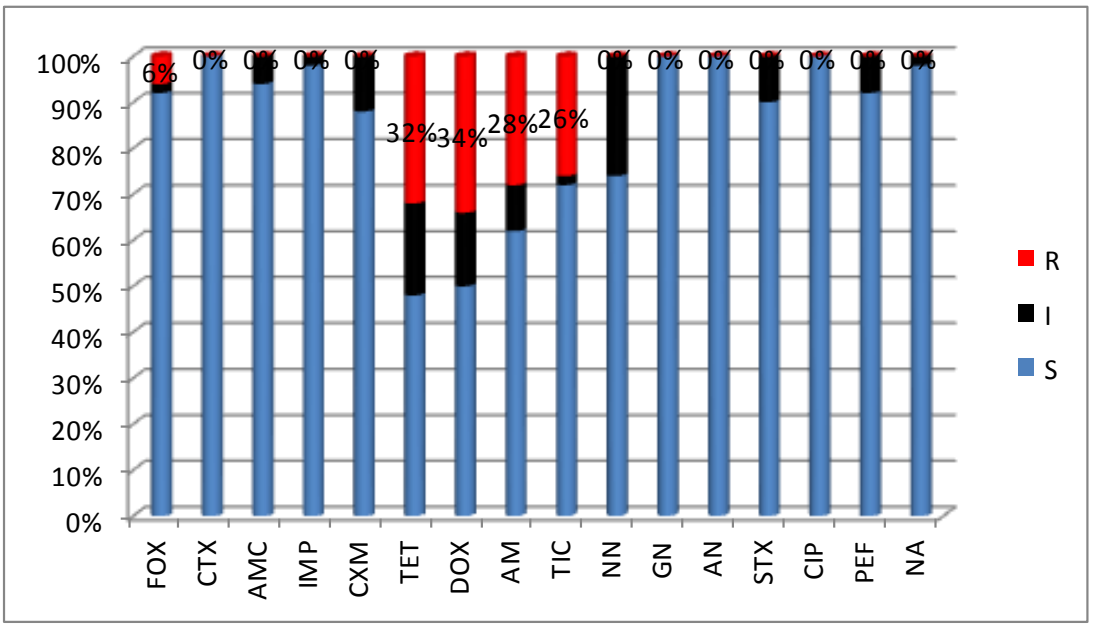


**Figure 1**. Graphic representation (%) of sensitivity / resistance to antibiotics in de *E. coli* strains isolated from drinking water (S = sensible, I = intermediate, R = resistant).

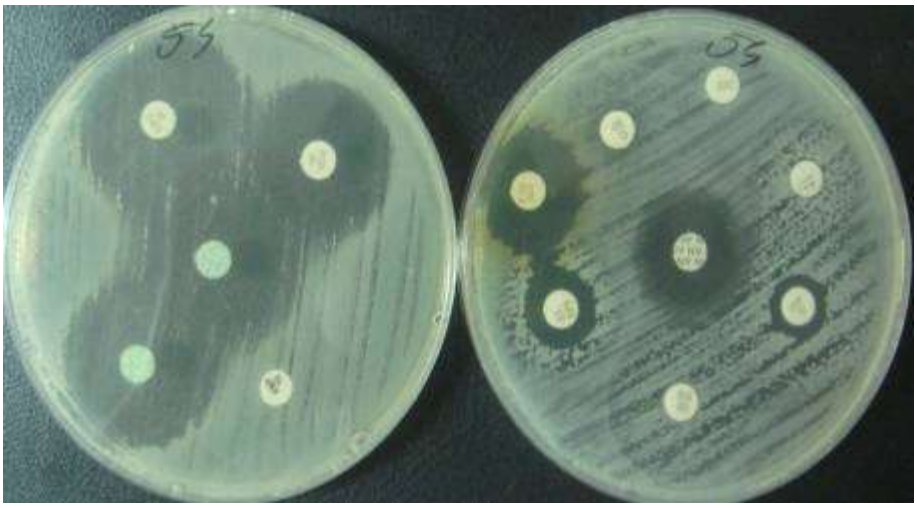

**Figure 2**. Antibiotic resistance phenotypes in *E. coli* strains isolated from drinking water (5 resistance markers).

**β-lactamase *screening* and confirmation tests**. 14% aquatic strains exhibiting resistance to β-lactam antibiotics proved to be positive for the presence of β-lactamases when tested by nitrocephine rapid test (fig. 3).

**Figure 3.** Appearance of β-lactamase positive strains in rapid chromogenic test to nitrocephine.

Plasmid DNA isolation was performed on 7 strains exhibiting multiple resistance markers. The presence of plasmid DNA was revealed in 33% of the tested strains. The isolated plasmids (1 to 3 strains) exhibited molecular weights ranging from 1.00 bp to 23 kbp (fig. 4).

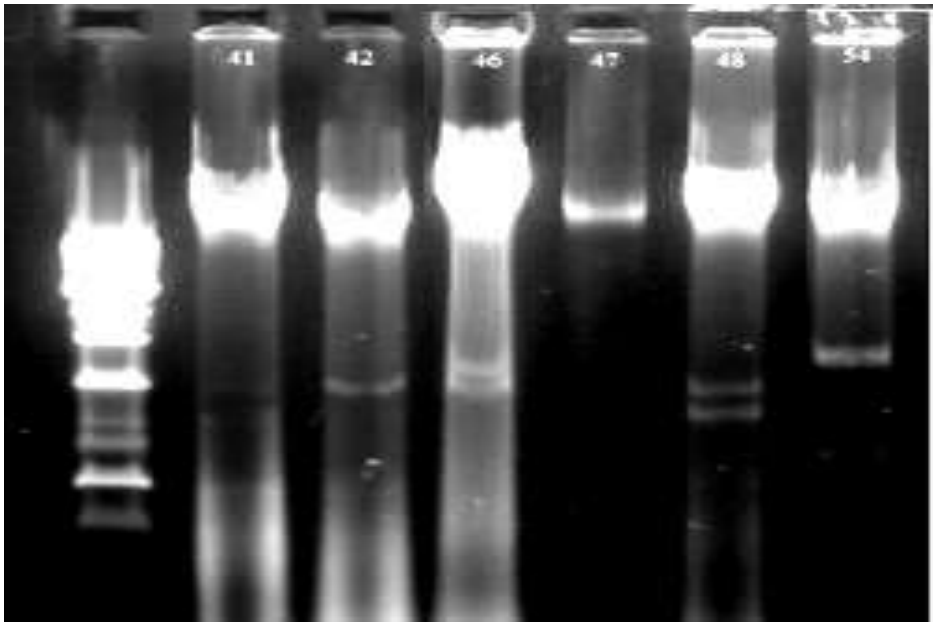


**Figure 4**. DNA profile migrated in 0,7% agarose gel, of *E. coli* strains isolated from drinking water.

## Results – virulence hallmarks

### Adherence to abiotic substratum

All tested strains *E.coli* strains showed high colonization ability of the inert substrate (represented by plastic) as demonstrated by the high positivity rate of slime test (fig. 5).

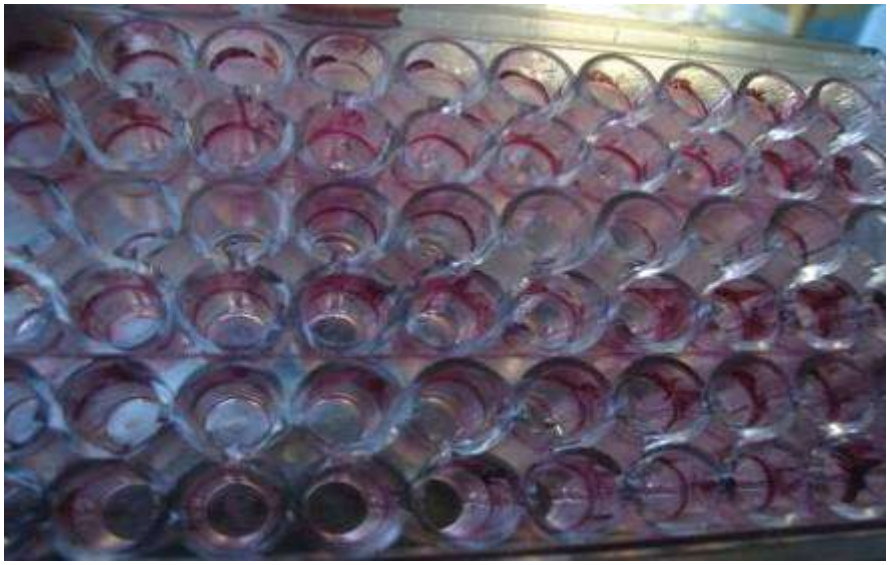

**Figure 5.** Representation of the experimental model used to test the capacity of the *E. coli* strains isolated from drinking water to colonize the inert substrate.

### Adherence to biotic substratum

Concerning the adherence to biotic substratum 90% of the strains isolated from drinking water exhibited high capacity of adherence to the cellular substrate (adherence indexes of 85-100% with localized, aggregative and diffuse patterns) (fig. 6) demonstrating the potential of these strains to colonize the animal and human tissues and to initiate an infectious process.

### Soluble enzymatic virulence factors

The tested strains poorly expressed soluble enzymatic virulence factors, i.e. the drinking water strains produced lipase, which could act as pore-forming toxin in case of tissue colonization and amylase, which could be implicated either in survival of strains in the external environment, or in the colonization (fig. 7).

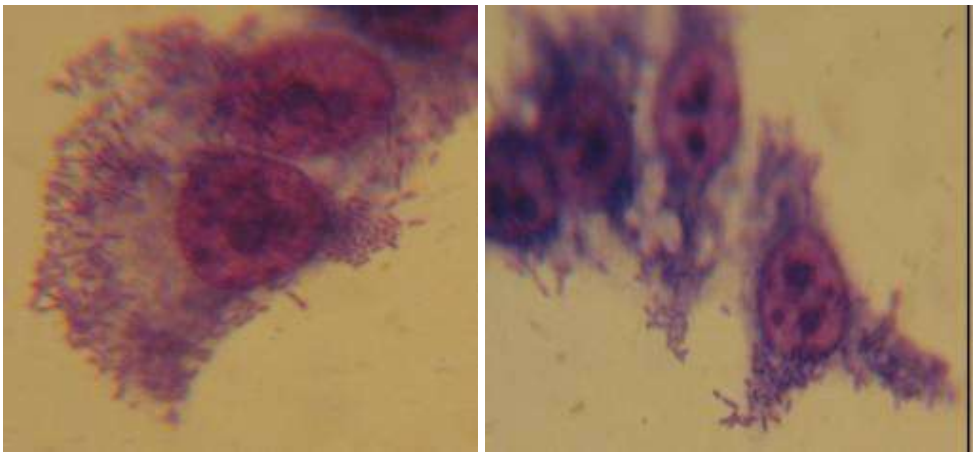

**Figure 6**.Diffuse adherence of *E. coli* strain to the HeLa cells (x2500, Giemsa staining)

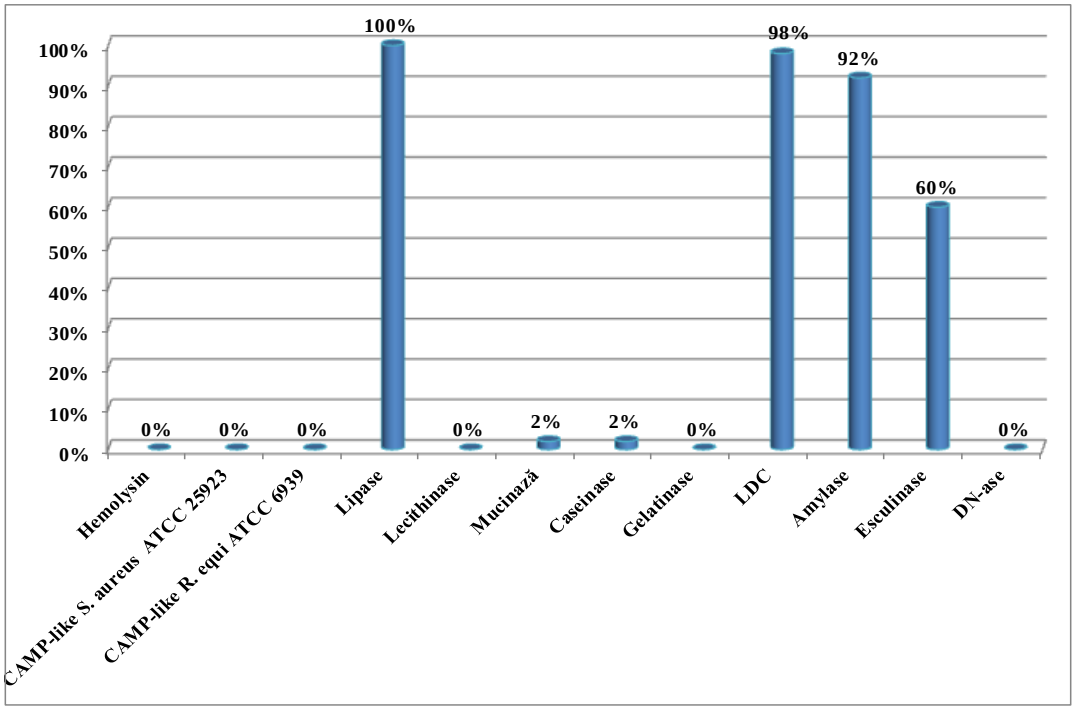


**Figure 7**. Graphic representation (%) the level of positivity of different soluble virulence factors in *E. coli* strains isolated from drinking water.

## Conclusions

Our study revealed that the *Escherichia coli* strains isolated from drinking water presented with resistance and multi-resistance profiles, correlated with the presence of some plasmids of different molecular weights. The concurrent resistance mechanisms to different classes of antibiotics could be mediated by the presence of nonspecific flow pumps. The high positivity levels of adherence to abiotic and biotic surfaces is pleading for the potential ability of these strains to colonize the human mucosal surfaces or implanted prosthetic devices and this to initiate and develop an infectious process, sustained by the secretion of soluble enzymes involved in virulence. The adherence pattern to the cellular substrate expressed most frequently was the localized one. The enzymes involved in the invasion and survival process were phenotypically expressed with higher frequencies than the pore forming enzymes, in all experimental conditions. The results of the present study have shown that aquatic medium signifies an appropriate ecological system for the existence and maintenance of a complex reservoir of antibioresistance and virulence factors with high risk for human host colonization and implications in the human health.

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# In vivo studies regarding the antioxidant effect of certain vegetal extracts on the stimulation of the antioxidant system in *Oncorhynchus Mykiss*, in conditions of overpopulation

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**Abstract**
The purpose of this paper was to evaluate the antioxidant effect of certain extracts obtained from indigenous plants (Allium ursinum and Alliaria petiolata) by introducing them in the diet of the rainbow trout reared in conditions of overpopulation in a recirculating system. At the end of the experimental period, the following investigations were accomplished: biochemical (MDA, TAC, GLU, TP, IgM), hematological (Ht, Hb, MetHb, E, VEM, HEM CHEM) and biotechnological (SGR, FCR). The highest significant values ($p<0.05$) of MDA, compared to the lots treated with vitamin E and Allium extracts, were registered in the muscles and liver of the fish in the witness lot not treated with extracts (the 2% version). In the liver, the significant increases of TAC, compared to the untreated witness lot, are in LotE ($p=0.023<0.05$), in LotAllium ($p=0.05\leq0.05$) and in LotAlliaria ($p=0.01<0.05$). The hemoglobin has values between 7.73-7.76 g/dL in LotAllium and between 7.55-7.62 g/dL in LotAlliaria, both being lower than LotE. The HEM values are comparable among the lots, ranging between 69.92-70.15pg, while the CHEM value is higher in LotMN, compared to LotE, LotAllium and LotAlliaria. The IgM has a minimum value of 88.23mg/dL in LotAlliaria (5%) and a maximum value of 92.03mg/dL in LotAlliaria (2%). In both experimental versions, positive correlations were obtained between the SGR and the average values of TAC in the analyzed lots. The final conclusion is that the vegetal extracts of Allium ursinum and Alliaria petiolata used in our experiments can determine, in certain concentrations, the reduction of the stress induced by overpopulation in Oncorhynchus mykiss.

**Abreviations: CHEM –** concentration of mean corpuscular hemoglobin, **E –** number of erythrocytes, **FCR –** Feed Conversion Ratio, **GLU –** glucose, **Ht** – hematocrit, **Hb –** hemoglobin, **HEM –** mean corpuscular hemoglobin, **Ig M –** immunoglobulin M, **MDA –** malondialdehyde, **MetHb –** methemoglobin, **PT –** total protein, **SGR –** Specific Growth Rate, **TAC –** Total Antioxidant Capacity, **VEM –** mean corpuscular volume.

**Keywords**: vegetal extracts, antioxidant effect, lipid peroxidation, rainbow trout, overpopulation.

## Introduction

Currently, the objective of the recirculating systems used in aquaculture is the intensification of the fish production which means, first of all, high population densities in the rearing basins. This aspect favors the maintenance of the culture biomass in conditions of oxidative stress, from the point of view of the ecophysiological comfort. The limited space in which the culture biomass is forced to live constitutes itself a factor that puts a heavy load on the adaptation mechanisms and which may lead to an immunity decline. Also, overpopulation influences the easy transmission and reproduction of specific pathogenic agents, which means the life conditions become dangerously altered.

Generally, the intensive rearing of animals, including of fish, involves large concentrations of individuals in a limited space. In intensive and superintensive aquaculture, there are rearing systems where the fish biomass can exceed 10-20 $Kg/m^3$ of water [18]. The increase of population density (overpopulation) in confined spaces reduces the vital space proportionally. The consequence is the appearance of neurohormonal modifications with direct implications on the biochemical and hematological values.

The importance of population density in fishery technology was emphasized by numerous authors who studied this aspect for different culture species and different production systems [4], [11], [12], [24-25].

The studies accomplished at international level on *Oncorhynchus mykiss* highlighted that this species, compared to other salmonidae, has few demands in terms of environmental conditions, has a good degree of feed assimilation and thus a fast growth rate in captivity. These technological issues represent an important argument for the rearing in superintensive recirculating system with the purpose of producing consumer trout.

Still, if we take into account the ecobiology of this species (the rainbow trout is a territorial fish), it is desired that the population density in the rearing basins should permit the establishment and maintenance of territories. In these conditions, overpopulation leads to stress. To support this hypothesis, it is accepted that fish agglomeration is a factor of chronic stress which may lead to behavioral and physiological modifications [27-28], [30], [43].

The necessity to obtain high fish productions in recirculating systems, corroborated with a reduction of the oxidative stress determined by overpopulation, stimulated the current research. Consequently, despite the increased progress registered over the recent years and the awareness of the complexity of such an approach made our study begin by the testing of vegetal extracts introduced in the feed of the rainbow trout and by the subsequent evaluation *in vivo* of the antioxidant effect through experimental biochemical, hematological and biotechnological analyses.

## Material and methods

The research took place in an aquarium-type superintensive recirculating rearing system, in the Laboratory of the Department of Aquaculture, Environmental Science and Land Register within the "Dunarea de Jos" University, Galati. Under the functional aspect the system was particularized to the technological demands for rearing culture fish species in limited spaces. The scheme of the recirculating system is presented in Figure 1.

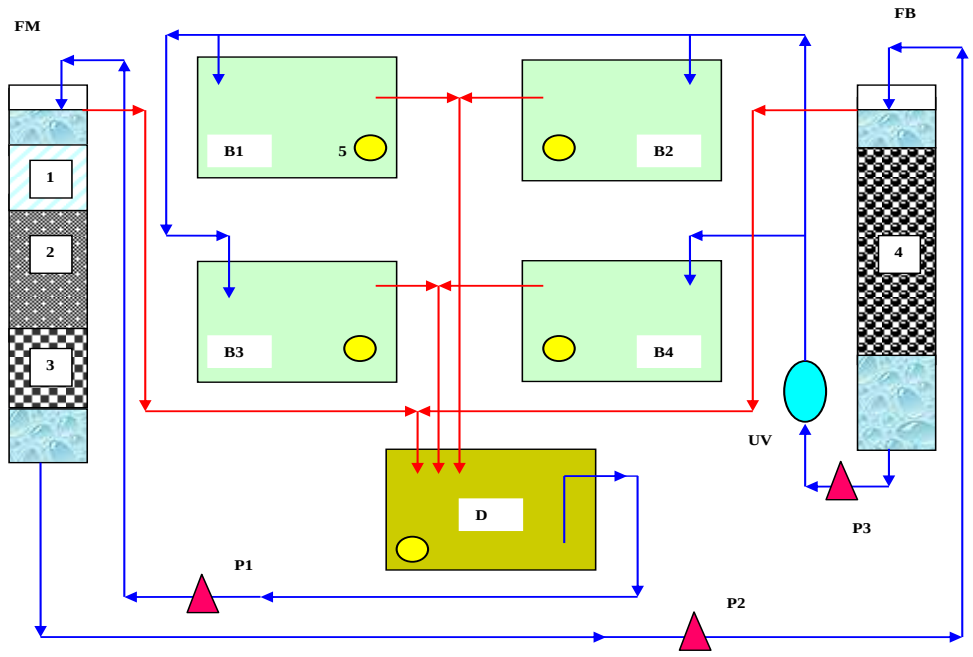


**Figure 1.** The scheme of the experimental recirculating system
Legend: $B_1$-$B_4$ –rearing units, D –decanting basin, $P_1$,$P_2$,$P_3$ .pumps, UV- sterilization lamp, FM- mechanical filter, FB-biological filter, 1-sponge, 2-sand, 3-gravel 4-bactobolts, 5- air nozzle.

The biological material was represented by the one-year-old culture species *Oncorhynchus mykiss*, reared in the same system during the juvenile stage. The biomass used in aquariums, considered admissible, which respected the bearing capacity of the recirculating system, but crucial in the accentuation of the oxidative stress, was 7300±60g /aquarium (73 fish/aquarium with average mass 100±5g/individual). The quantity of feed administered (ration) was 3% of the initial biomass. The feeding frequency was established to twice a day (at 9 a.m. and 5 p.m.). The feed was administered in the form of extruded pellets (Nutra Pro

MP-T), sized 1.7 mm and with the following biochemical composition: crude proteins 50%, crude fats and oils 20%, fibers 10%, crude ash 8%, phosphorus 1.2%, calcium 1%, sodium 0.4%, vitamin A 6000 UI/kg, vitamin $D_3$ 1200UI/kg. As antioxidant-**known witness-** vitamin E (α-tocopheryl acetate) was used as soft capsules produced by S.C. Biofarm S.A.

Two experimental versions were accomplished. Duration 15 days/version:

**-version a**: plant addition 2% in $Lot_{Allium}$ and $Lot_{Allium\ Alliaria}$; $Lot_{Allium MN}$; $Lot_{Allium\ E}$.

**-version b**: plant addition 5% in $Lot_{Allium\ Allium}$ and $Lot_{Allium\ Alliaria}$; $Lot_{Allium\ MN}$, $Lot_{Allium\ E}$.

**Legend**: $Lot_{Allium\ MN}$ – untreated witness lot, $Lot_{Allium\ E}$ – lot treated with vitamin E (50mg/kg biomass/day).

***Preparation of the feed, collection and analysis of the biological samples***

The extracts were obtained by double maceration [44] in alcohol 70° for 15 days at room temperature from the two plants – *Allium ursinum* and *Alliaria petiolata* (leaves + flowers in equal ratio for each plant) and vitamin E. They were incorporated into the feed after a previous evaporation of the alcohol in Rotavapor by homogenization with gelatin 2%. The drying of the feed was done in the drying oven, at 27°C for 12 hours for the adhesion of the extracts and of vitamin E to the feed surface. Before collecting the biological samples (blood and tissues), the trout individuals were anesthetized. The anesthetic used was a solution of 2-phenoxyethanol (1ml for 3 liters of water) in which the fish were immersed for 1 minute. The method we used was collection from the caudal vein in the following stages: tamponing the punctured area with betadine 10% (in order to avoid contaminating the blood with water or mucus), introduction of the syringe needle on the direction located behind the anal fin, puncturing the caudal vein and collection of approx. 1.5-2 ml of blood. The collected blood was poured: a part of it (1ml) in Ependorf tubes with anticoagulant (heparine) for hematological determinations and indicators of oxidative stress, and another part without coagulant for biochemical determinations in other Ependorf tubes.

The harvesting of the internal organs (liver, heart, kidneys, muscles, intestine, gills) was accomplished by dissection, collection and weighing on the analytical balance. The blood samples without heparine were centrifuged for 10 minutes at 3500 rotations/minute and the plasma was separated for the biochemical determinations and for the indicators of the oxidative stress. Of the total amount of blood, 0.1 ml were used immediately for the determination of methemoglobin.

The biochemical determinations were accomplished in the chemistry laboratories within the Faculty of Sciences from "Dunarea de Jos" University, Galati.

The analysis of lipid peroxidation was accomplished by the determination of the concentration of **malondialdehyde** (MDA) and of other substances which

react with the thiobarbituric acid (TBARS), with the help of the method described by Draper and Hadley [7]. The determination of the **total antioxidant capacity** (TAC) from the blood plasma and the tissues of the analyzed samples was done by the method described by Re [31] and Van Den Berg [39]. The quantitative determination of **hemoglobin** was accomplished through the use of the colorimetric method using the Sahli hemoglobinometer and the 0.02 dropper for hemoglobin. This method is based on the fact that hemoglobin mixed with hydrochloric acid turns into hematin hydrochloride. The determination of the **hematocrit** or the volume percentage of erythrocytes, which represents the concentration of erythrocytes in 100 ml of whole blood, was accomplished by the microhematocrit method, which involves centrifuging the blood in capillary tubes (30µL) of heparinized microhematocrit. The determination of **the number of erythrocytes** (red cells) was accomplished according to a classical method using the hemocytometer (the Neubauer counting chamber) and dilution liquid.

The calculation formula used for the determination of the number of erythrocytes, with correction of surface, height and dilution, expressed in millions of cells (e.g. $10^6/mm^3$) is as follows:

$$\mathbf{X} = \frac{400 \times 200 \times N}{80} = \mathbf{10000} \times \boldsymbol{N},$$

where:
**N**-counted erythrocytes in 80 (16x5) squares;
**4000**-the volume of squares with 1/20mm side;
**200**-blood dilution.

The erythrocyte constants reflect the working degree of the erythrocytes, the content of hemoglobin in each erythrocyte and the ratio between this content and its value. The most important erythrocyte constants are:

- The mean corpuscular volume (**VEM**) represents the mean volume of the erythrocyte. The values obtained vary according to age and sex. They are expressed in $\mu m^3$ and are calculated according to the formula:

$$VEM = \frac{Ht \times 10}{E}$$

where: **Ht** is the hematocrit (%) ;
**E-**is the number of erythrocytes ($mill/mm^3$) in blood

- The mean corpuscular hemoglobin (**HEM**) represents the mean content of hemoglobin of the erythrocytes. It is expressed in picograms (pg) which represent $10^{-12}$ g and are calculated according to the formula:

$$\mathbf{HEM} = \frac{\boldsymbol{Hb} \times \mathbf{10}}{\boldsymbol{E}}$$

where: Hb is the hemoglobin in grams/100 ml of blood. It is the number of erythrocytes expressed in mill./mm$^3$ of blood.

- The concentration in mean corpuscular hemoglobin (**CHEM**) represents the ratio between the corpuscular hemoglobin and its volume, being the most truthful erythrocyte constant. It is expressed in grams/dl of blood and is calculated according to the formula:

$$\mathbf{CHEM} = \frac{\boldsymbol{Hb} \times \mathbf{100}}{\boldsymbol{Ht}}$$

where:

**Hb** is the hemoglobin expressed in grams/100 ml of blood;

**Ht** is the hematocrit (%).

At the end of the experimental period/**version**, the fish were weighed and the bioindicators of technological performance were calculated, namely:

- **The specific growth rate** (**SGR**) was calculated according to the method described by Hevroy et al. [10]:

$$\mathbf{SGR=(\ln W_t - \ln W_0) \times 100 \times t^{-1}\ (\%BW/day),}$$

where,

$\mathbf{W_t}$- final biomass; $\mathbf{W_0}$ –initial biomass; **t**-experiment duration (days), **BW**-body weight (g);

- **The feed conversion ratio** (**FCR**) was calculated according to the method described by Shalaby et al. [33]:

$$\mathbf{FCR= Q/ S_r\ , (g/g),}$$

where:

**Q**-the quantity of feed administered (g) ; $\mathbf{S_r= W_t - W_0}$ (g).

**The statistical analysis of the experimental data**

The results were expressed as means± and standard deviation (SD). The statistical data were analyzed in the Windows 2007 programs (Microsoft Office Excel, Microsoft Office Word), while the means and standard deviations were calculated according to standard methods for all parameters.

The Student "t" test was used for the comparison of the means. The difference between two means was considered significant for a value of $p<0.05$.

**Results and Discussions**

Over the 15 days of experiments, the fish with identification chips from the experimental lots had a slightly agitated behavior, but without pathological symptoms or the presence of parasites on their tegument. We must emphasize that the physic-chemical parameters of the water were maintained in the optimum values for the rearing of this species so as not to create additional stress for them.

If we look at Figure 2, we can see that the level of lipid peroxidation induced by the overpopulation stress is more accentuated for the muscular and liver tissue in the witness lot (a lot not treated with vitamin E or vegetal extracts obtained from *Allium* and *Alliaria*). It seems these tissues are the most sensitive to the overpopulation stress in the rainbow trout.

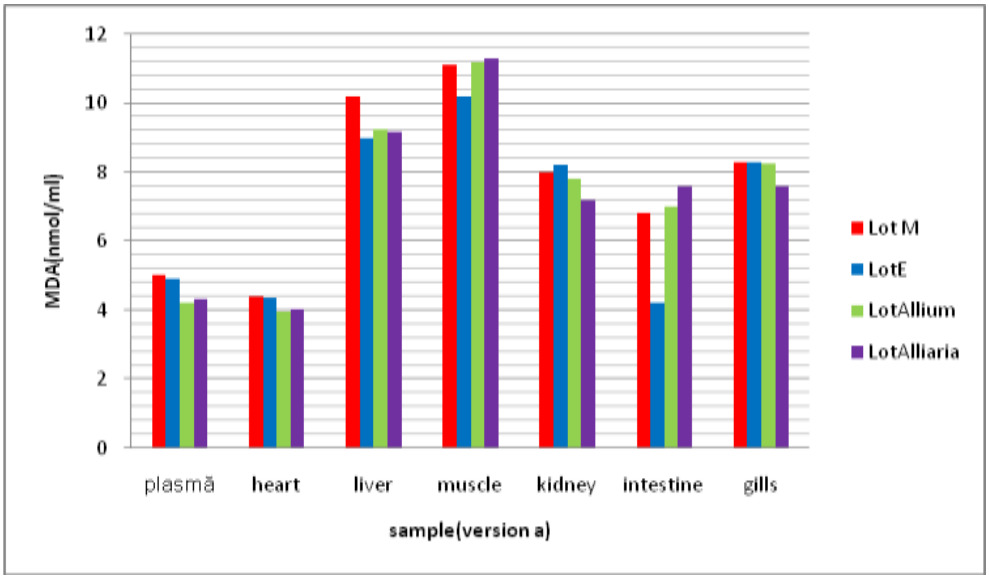


**Figure 2.** The dynamics of the malondialdehyde index (MDA) from the tissue and blood plasma of fish in version **a** (plant extract 2%)

In what regards the treatment with extracts obtained from *Allium ursinum* and *Alliaria petiolata*, given the overpopulation stress, the reduction of the MAD tissue level is significant ($p<0.05$) for the liver, both in the 2% concentration and in the 5% concentration of the vegetal extract (see Figure 2 and Figure 3).

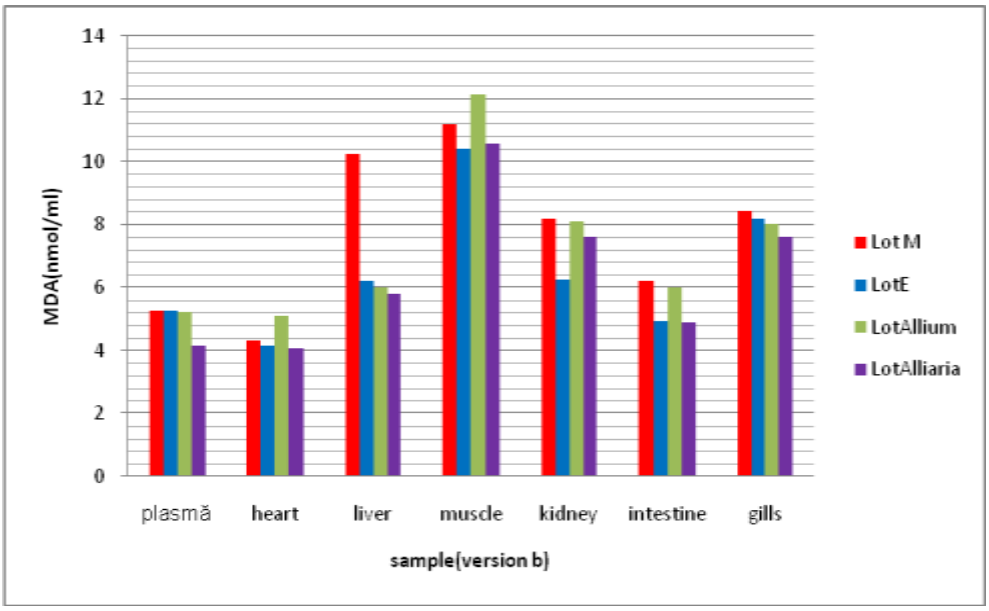


**Figure 3.** The dynamics of the malondialdehyde index (MDA) from the tissue and blood plasma of fish in version **b** (plant extract 5%)

In the case of the blood plasma, the heart, muscle, intestine, gills and kidneys, the treatment with vegetal extract 2% from both plants (*Allium* and *Alliaria*), given the overpopulation stress, does not reduce the level of lipid peroxidation (expressed by the MAD tissue concentration (see Figures 2 and 3). Vitamin E either does not reduce the level of lipid peroxidaton (increased by overpopulation stress) in the same organs.

Similar results were obtained by Metwally [17], who followed the MDA evolution in the crustacean *Litopenaeus vannamei* over a period of 84 days. Indeed, the vegetal extracts introduced in the shrimps diet led to the reduction of the MDA concentration in gills, first insignificantly (at 21 days), and then significantly (at 84 days).

Regarding the total antioxidant capacity, the 2% extract of *Allium* and *Alliaria* increases this capacity, given the overpopulation stress in the rainbow trout, for blood plasma, muscles, and liver. Moreover, the 2% extract of *Alliaria* increases the same antioxidant capacity also in the case of the renal tissue (see Figure 4, version a).

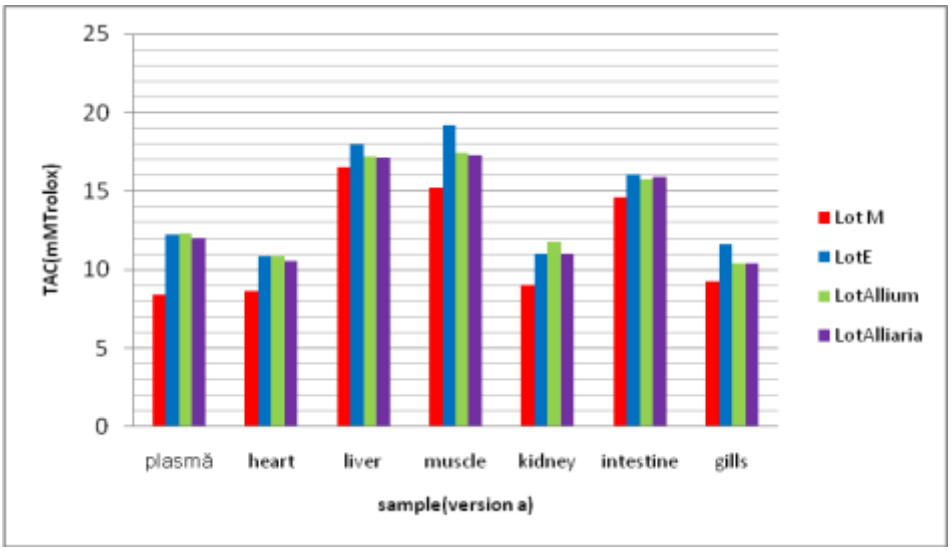


**Figure 4.** The dynamics of the total antioxidant capacity in tissues and blood plasma of fish in version a (plant extract 2%)

Given the overpopulation stress, the treatment was done with the 5% extract of *Alliaria petiolata*, and there was an increase of the total antioxidant capacity in the case of the blood plasma, heart, liver and muscle as well, compared to the untreated lot (see Figure 5).

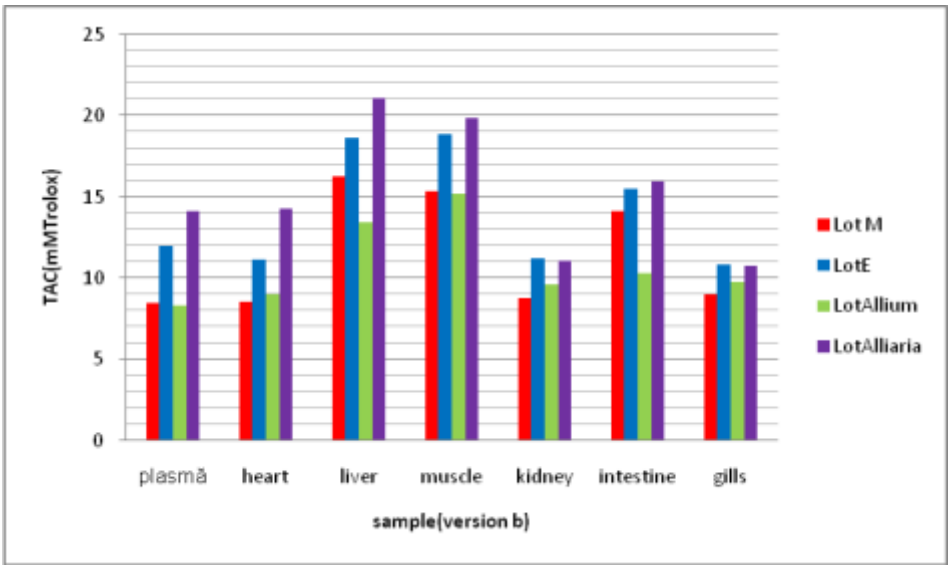


**Figure 5.** The dynamics of the total antioxidant capacity in tissues and blood plasma of fish in version b (plant extract 5%)

Given the overpopulation stress, vitamin E determined an increase of the total antioxidant capacity in plasma, liver and muscles (experimental version a), or in liver and intestine (experimental version b; see Figure 5). If we trace an overview of the experimental results where we followed the effect of the 2% and 5% vegetal extracts from *Allium* and *Alliaria* in the rainbow trout subjected to overpopulation stress, we observe that the vegetal extracts were at least equal to vitamin E in terms of stimulating the total antioxidant capacity (see Figures 4 and 5).

These results allow us to appreciate the antioxidant effect of the used extracts, which can be attributed to the content in bioactive compounds such as: flavonoids, volatile oils, vitamins and others. In support of this discovery, some authors attributed to vitamin C an indirect antioxidant effect [21], [37], while others signaled a positive, modulating effect of vitamins C and E in response to stress [9], [13], [22], [32], [35].

For a more complete evaluation of the tested vegetal extracts in the attenuation of the induced oxidative stress, in the present experiment we also followed the evolution of certain hematological parameters. In this regard, for the appreciation of the physiological state of the fish, we accomplished the hemoglobin dosing, considered a precise and quick test for the checking of the hematological homeostasis [18]. Hemoglobin is the respiratory pigment with important role in the increase of the blood's capacity to transport oxygen. In our experiment (see Table 1), we observed that vitamin E, administered in conditions of overpopulations stress in the rainbow trout, determines modifications of the hemoglobin content and number of erythrocytes (comparison between $Lot_E$ and $Lot_{MN}$; MN = untreated witness).

**Table 1**

Variation of the hematological parameters in fish at the end of the experiment

| Hematological parameter | $Lot_{MN}$ | $Lot_E$ | $Lot_{Allium}$ | | $Lot_{Alliaria}$ | |
|---|---|---|---|---|---|---|
| | | | 2% | 5% | 2% | 5% |
| **Ht (%)** | 41±5.23$^{b}$ | 40±3.38$^{b}$ | 36±6.22$^{b}$ | 32±6.99$^{b}$ | 36±3.89$^{b}$ | 38±5.35$^{b}$ |
| **Hb(g/dL)** | 6.72±0.10$^{ab}$ | 7.80±0.08$^{a}$ | 7.76±0.07$^{a}$ | 7.73±0.89$^{b}$ | 7.62±0.58$^{b}$ | 7.55±0.68$^{b}$ |
| **No.E x$10^6$/$mm^3$** | 1.05±0.03$^{ab}$ | 1.22±0.04$^{b}$ | 1.28±0.02$^{a}$ | 1.12±0.11$^{b}$ | 1.29±0.03$^{a}$ | 1.27±0.11$^{b}$ |
| **VEM(µ$m^3$)** | 365.10±12.5$^{b}$ | 420.33±10.6$^{b}$ | 385.17±24.0$^{b}$ | 386.55±15.2$^{b}$ | 419.31±10.7$^{b}$ | 421.04±20.1$^{b}$ |
| **HEM(pg)** | 70.15±4.48 | 70.02±3.07 | 69.95±7.29 | 69.90±9.02 | 69.97±4.80 | 69.92±5.83 |
| **CHEM g/dL** | 18.46±2.0$^{b}$ | 18.50±1.03$^{b}$ | 18.90±1.92$^{b}$ | 18.58±2.11$^{b}$ | 18.58±3.41$^{b}$ | 18.49±1.47$^{b}$ |

The various superscript letters in the columns indicate significant($^{a}$) and insignificant($^{b}$) differences with cu $p<0.05$; the statistical analysis was accomplished on the horizontal row thus: $Lot_{MN}$ compared to $Lot_E$ and the lots not treated with the extracts.

The vegetal extracts of *Allium ursinum* and *Alliaria petiolata* influence the level of the hematological parameters. Thus, the *Allium* extract in 2% concentration determines the increase of hemoglobin and the number of erythrocytes, while the *Alliaria* extract in 2% concentration only increases the number of erythrocytes (see Table 1). The increase in the number of erythrocytes, and thus of the quantity of hemoglobin in the lots treated with *Allium* and *Alliaria* extracts could be the result of the direct action of the active principles from these extracts on the level of production of the figurate elements. As we have shown in the experiment where the stress was induced by intoxication with sodium nitrite, any state of stress is associated with more intense energy consumption at the level of cells and tissues, because only this way the cells and tissues can efficiently resist metabolically to the state in which they are.

So, our analyses emphasize the increases of hemoglobin in the lot with vitamin E addition, but also in the lots with addition of vegetal extracts. This means that a larger quantity of oxygen transported to the tissues where the intensified oxidative metabolic processes and production of energy ensure their increased resistance to stress. In some research, it is shown that the significant reduction of the amount of hemoglobin in blood can affect the amount of oxygen for tissues and the result is the slowing down of the metabolic rate, thus a reduced production of energy [1]. Also, the significant reduction of the amount of hemoglobin can be caused by the increase of the rate of hemoglobin destruction, or the reduction of the rate of its synthesis, given the state of stress. According to other authors [23], these hemoglobin modifications are determined by different forms of stress which in turn determine the rapid increase of the hemoglobin concentration due to the recruiting of erythrocytes from the spleen and the hemoconcentration installed as a result of the loss of plasmatic water.

The hematocrit (the volume percentage of erythrocytes) does not modify significantly in the lot with addition of vitamin E, as in the lots treated with extracts of *Allium ursinum* and *Alliaria petiolata*, compared to the witness lot ($Lot_{MN}$). According to some research [23], the increase of hematocrit in fish is joined by an increase of blood viscosity, which is considered the superior limit of the strategy of adaptation to stress. Other authors [15], [42] remarked the influence of different stress factors on the hematocrit in certain culture species, which determined a dynamics of the hematocrit in a very wide range, between 20-40%.

The slight decrease of the hematocrit joined by an increase of the hemoglobin influenced the increase in the number of erythrocytes in $Lot_{E}$, $Lot_{Allium}$ and $Lot_{Alliaria}$. The data in the specialized literature account for the fact that in *Oncorhynchus mykiss* the number of erythrocytes can vary from 0.85 to 1.50($x10^6/mm^3$) depending on age, sex, physiological state etc. The values obtained by us fit in the superior limit, with the highest value in $Lot_{Alliaria}$ (2%)

and $Lot_{Allium}$ (2%). These values are significantly increased ($p<0.05$), compared to the untreated witness lot.

Similar results were obtained by Valenzuela et al. [38] in a study regarding the effect of physical stress induced by the increase of temperature and continuous application of light, which led to the increase of hemoglobin concentration and number of erythrocytes.

The analysis of the erythrocyte constants (VEM, HEM, CHEM) can help to detect the presence of physiological ailments in the process of hemoglobin formation and offers information regarding the size, shape and hemoglobin load of erythrocytes.

Under the influence of the vegetal extracts and vitamin E, the constant erythrocyte values from the analyzed samples registered the following modifications:

- The mean corpuscular volume (VEM) increased insignificantly ($p>0.05$) in the analyzed lots compared to the untreated witness lot;
- The mean corpuscular hemoglobin (HEM) registered relatively constant values, compared to $Lot_{MN}$;
- The concentration of mean corpuscular hemoglobin (CHEM) increased insignificantly ($p>0.05$), compared to the untreated witness lot.

The analysis of the values of the three erythrocyte constants allow us to formulate the observation that the VEM, HEM and CHEM fit in the limits considered optimal for the rearing of salmonidae. However, we remark that the values in the inferior limit are found in the witness lot. Also, the values of the erythrocyte constants in the lots fed with vegetal extracts are comparable with those of the lot treated with vitamin E.

The present study continued with the analysis of biochemical parameters of blood: glycemia, total plasma proteins and plasma immunoglobulin M. In the case of our experiment, where the stress was induced by overpopulation in the rainbow trout, we observed that neither the vegetal extracts of *Allium ursinum* and *Alliaria petiolata*, nor vitamin E modify significantly the glycemia level compared to $Lot_{MN}$.

In other words, we can say that in conditions of overpopulation stress, neither the vegetal extracts of *Allium ursinum* and *Alliaria petiolata*, nor vitamin E enhance the mechanisms of glucose release from storage in order to be consumed by the peripheral tissues. It is possible that the overpopulation stress should be easier to bear by the fish organism, compared to the stress induced by intoxication with sodium nitrite (unpublished personal data), as the fish cope more easily with this type of stress without additional energy consumption (see Table 2).

The plasma sugars represented especially by glucose are normally found at values between 40-90mg/100mL in the fish blood, while in the healthy rainbow trout, they range around 71mg/100mL of blood [12]. According to some authors,

glycemia can be influenced by a number of technological factors and constitutes an important marker for the stress states in fish. The manipulation stress can lead to a significant increase of the glycemia up to values of 137mg/dL [2]. Other authors state that an increase in glycemia represents the response of the organism in fish exposed to acute or chronic stress [19-20], [41], [3], [29], [36].

**Table 2**

Biochemical parameters in fish in conditions of overpopulation

| Sample | Statistical indexes | GLU mg/dL | TP g/dL | Ig M mg/dL |
|---|---|---|---|---|
| **$Lot_{MN}$** | X±ES | 120.2±10.01 | 3.42±0.22 | 90.62±4.06 |
| | n | 4 | 4 | 4 |
| **$Lot_{E}$** | X±ES | 112.0±8.48 | 4.30±0.08 | 89.00±3.22 |
| | n | 4 | 4 | 4 |
| | ±M% | -6.82 | +25.73 | -1.79 |
| **$Lot_{Allium}$** | plant concentration 2% | | | |
| | X±ES | 87.20±3.19 | 4.25±0.23 | 90.1±1.44 |
| | n | 5 | 4 | 5 |
| | ±M% | -27.45 | +24.27 | -0.57 |
| | plant concentration 5% | | | |
| | X±ES | 89.13±6.03 | 3.15±0.31 | 91.6±0.2 |
| | n | 5 | 5 | 5 |
| | ±M% | -25.84 | -7.9 | +1.08 |
| **$Lot_{Alliaria}$** | plant concentration 2% | | | |
| | X±ES | 87.77±7.22 | 3.60±0.87 | 92.03±5.07 |
| | n | 5 | 5 | 5 |
| | ±M% | -26.98 | +5.26 | +1.5 |
| | plant concentration 5% | | | |
| | X±ES | 85.94±5.80 | 4.46±0.44 | 88.23±1.20 |
| | n | 4 | 5 | 5 |
| | ±M% | -28.50 | +30.41 | -2.6 |

**Legend**: $Lot_{M}$-witness lot, $Lot_{E}$ (addition of vitamin E), $Lot_{Allium}$ (plant addition), $Lot_{Alliaria}$ (plant addition) X±ES-standard deviation, n-number of samples, ±M%-percentage difference between the analyzed lot and the untreated witness lot.

If we refer to the plasma proteins and immunoglobulin M, the results of our experiments show that neither the vegetal extracts of *Allium ursinum* or *Alliaria petiolata*, nor vitamin E modify significantly this blood parameter in a stress state induced by overpopulation. At least from the point of view of the biochemical parameters studied, we can say that stress induced by intoxication with sodium nitrite (unpublished personal results) is more difficult to bear by the organism of the rainbow trout compared to the stress induced by overpopulation.

According to some authors [34], the normal values of the total serum proteins in fish range between 3.5 and 5.5g/dL. In *Oncorhynchus mikiss,* the optimal values published in specialty articles are different: 3.64-3.80 g/dL [14]; 2.52-3.16 g/dL [16]; 2.92 g/dL [40]; 3.7-4,7 g/dL [5]. Still, these can display ample variations according to species, age, sex, water temperature, season, quality and quantity of feed [26].

The proteinemy in our samples is slightly increased compared to the lot not treated with vegetal extracts with percentage rises between 5.26-30.41%, with one exception in $Lot_{Allium}$ (5% extracts concentration), where the protein level decreases by 7.9%. The explanation we propose is that the variations of plasma proteins may be due to the intensification of the appetite manifested in the fish in $Lot_{Alliaria}$, $Lot_{E}$ and $Lot_{Allium}$ (2%). This phenomenon may be due to the increased protein content in the feed (50% crude protein), associated with the intake of antioxidants in the vegetal extracts tested. The 5% *Allium ursinum* concentration from the extracts used proved to be less agreeable and thus less assimilated by the fish. The increase of the protein level in blood was also signaled by Farahi et al. [8], who added garlic (*Allium sativum*) in the fish feed.

In the ontogenetic development, the first class of immunoglobulin which appears is the immunoglobulin M class (IgM) and they represent the antibodies that appear after the first contact with the antigen. The IgM values can be influenced by a number of exogenous and endogenous factors and range between 1-100mg/dL [26]. The values obtained by us register both increases and decreases compared to the witness lot not treated with vegetal extracts. The most considerable percentage decrease (2.6%) was registered in $Lot_{Alliaria}$ (5%), while the increases were of +1.8% la $Lot_{Allium}$ (5%). All the values fit in the superior limit of the optimum range, without significant statistical differences. Most likely, the stress induced by overpopulation triggers the organism's reaction to this type of stress, but within the normal values.

The study of growth performance by means of the main biotechnological indicators (see Table 3) represents a way to appreciate quantitatively the normality degree of the physiological state of the fish organism. This aspect allowed us to evaluate the dynamics of the culture biomass by the analysis of the specific growth rate (SGR) and of the feed conversion factor (FCR). The addition of vitamin E in the fish feed led to an increase of the SGR by 42.5% compared to the witness lot not treated with vegetal extracts. The vegetal extracts added to the feed were tolerated by the fish differently, a fact which was noticed both during the experiment and after the analysis of the first samples. The highest percentage increases of the SGR, compared to $Lot_{MN}$, were registered in $Lot_{Alliaria}$ (5%), comparable to $Lot_{Allium}$ (2%).

Similar results were obtained in other research as well [8], such as different amounts of *Allium sativum* being introduced in the diet of the species *Oncorhynchus mykiss*, or common sea-buckthorn in the feed of carp [6].

**Table 3**

Biotechnological indicators in the rainbow trout in conditions of overpopulation

| **Biotechnological indicators** | | **$Lot_{MN}$** | **$Lot_{E}$** | **$Lot_{Allium}$** | **$Lot_{Alliaria}$** |
|---|---|---|---|---|---|
| **version a plant concentration 2%** | | | | | |
| FCR (g/g) | | 2.28 | 1.54 | 1.52 | 1.44 |
| SGR | %BW/day | 1.2 | 1.71 | 1.73 | 1.81 |
| | % | 100 | 142.5 | 144.17 | 150.83 |
| **version b plant concentration 5%** | | | | | |
| FCR (g/g) | | 2.31 | 1.85 | 2.19 | 1.41 |
| SGR | %BW/day | 1.19 | 1.45 | 1.24 | 1.84 |
| | % | 100 | 121.84 | 104.2 | 154.62 |

Legend: FCR- Coefficient of feed conversion; SGR- Specific growth rate

If we take into account the specific growth rate, corroborated with the feed conversion factor and the 98.75% level of fish survival, our results are optimistic. Our study shows that the feed supplementation with vegetal extracts leads to the increase of the total antioxidant capacity and the decrease of the lipid peroxidation level. These changes of the antioxidant status are positively correlated with the specific growth rate (see Figure 6).

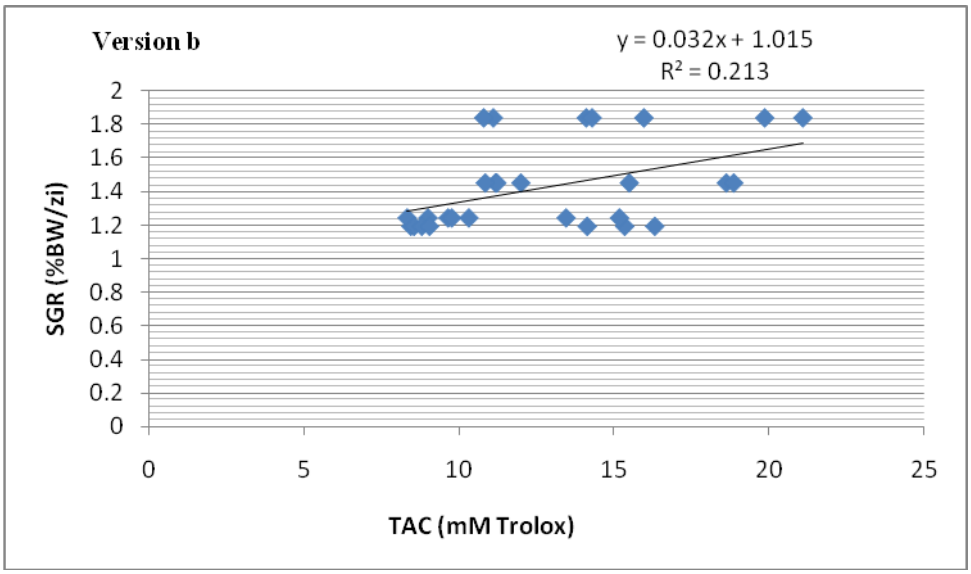


**Figure 6.** Correlation between the specific growth rate (SGR) and the total antioxidant capacity (extract concentration 5%, n=28)

Based on the experimental data obtained, we can state that the addition of vegetal extracts from *Allium ursinum* and *Alliaria petiolata* in the feed helps to maintain the balance between oxidants and antioxidants, preventing membrane deteriorations caused by oxidative stress.

**Conclusions**

1. The *Allium ursinum* and *Alliaria petiolata* extracts have antioxidant properties in certain concentrations;
2. The antioxidant properties of the vegetal extracts used are comparable to those of vitamin E (used as known antioxidant);
3. The intensity of the stress induced by various free radicals, including those of oxygen, appreciated according to the malondialdehyde (MDA) value, is different in the analyzed organs;
4. Overpopulation triggers an increase of lipid peroxidation, especially in liver and muscles;
5. The tissue level of malondialdehyde depends on the nature of the extract. The vegetal extracts from both species are comparable as antioxidant effect in **version a** (concentration 2%);
6. The *Alliaria petiolata* extract (concentration 5%) influences positively the total antioxidant capacity, compared to the untreated witness lot (standard feed) and with $Lot_E$ (addition of vitamin E) ;

7. The erythrocyte fragility is correlated with the intensity of stress induced by free radicals, including oxygen free radicals, in the lot not treated with vegetal extracts or vitamin E (considered by us witness lot);
8. The tested vegetal extracts favor the increase of hemoglobin level in blood, within physiological limits;
9. It was observed that neither the vegetal extracts from *Allium ursinum* and *Alliaria petiolata* nor vitamin E modify significantly the glycemia level compared with the untreated witness lot;
10. What results from the analysis of the biotechnological indicators is an active feeding of the fish in case of **version a** (2% extracts) and differentiated in the case of **version b** (5% extracts).
11. Consequently, after the installation of oxidative stress by overpopulation, it was observed that the treatment with extracts from *Allium ursinum* and *Alliaria petiolata*, leads to an attenuation of the level of lipid peroxidation and to an enhancement of the total antioxidant capacity.

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