Plant-based and immunostimulant-enhanced diets modulate oxidative stress, immune and haematological indices in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The aim of this study was to investigate the effects of three commercial diets, standard, immunostimulant-supplemented (β-glucan, vitamins C and E) and plant-based, on the degree of oxidative stress in tissues of rainbow trout (*Oncorhynchus mykiss*). Selected immune and haematological indices were measured and challenge with *Aeromonas salmonicida* was conducted. The plant-based diet systemically modulated all oxidative stress biomarkers (thiobarbituric acid reactive substances; reduced and oxidised glutathione and their ratio) in the intestine. The ratio was elevated in all organs (liver, kidney, muscle and intestine) and an enhancement of respiratory burst and complement activity was observed even in the control fish. With the standard diet, an elevation of thiobarbituric acid reactive substances in the intestine and a decrease in some immune indices appeared after challenge. Less distinctive changes and the lowest mortality rate (the highest being with the plant-based feed) were obtained with immunostimulants. Increased total immunoglobulin, relative lymphocytosis and a decrease in the phagocyte count were observed. This study contributes especially by a thorough examination of oxidative stress indices in different tissues. The exact composition of these commercial diets is a trade secret; however, knowledge of their effects is extremely important for fish farmers using them; therefore, this study has a great practical impact.

Aeromonas salmonicida, glucan, glutathione, respiratory burst, TBARS

Fish in intensive aquaculture systems are often exposed to high levels of stress, making them particularly vulnerable to disease and increasing oxidative stress. In recent years, efforts have been made to reduce the amount of antibiotics used in raising livestock, including fish farming, and alternative ways of increasing the resistance of susceptible fish have been investigated. As a preventive measure, immunostimulants can easily be administered in the form of dietary supplements. A wide range of biological and synthetic substances has the ability to modulate the fish immune system, with the most widely used comprising polysaccharides, herbs, microorganisms and nutrients such as vitamins (Wang et al. 2017). Micronutrients, along with macronutrients and probiotics, play an important role in animal welfare by maintaining the organism's oxidative balance (Enes et al. 2012). It should always be remembered, however, that individual feed supplements can interact (Puangkaew et al. 2004).

In recent years, the growth of aquaculture has led to the frequent substitution of fish meal with plant-based feeds that are more economically sustainable. However, in carnivorous

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fish, including rainbow trout (*Oncorhynchus mykiss*), these feeds do not correspond to their natural diet. In a previous study, Le Boucher et al. (2011) were able to show a significant reduction in the growth rate of rainbow trout fed a plant-based feed, without a negative impact on the survival rate of the fish.

The addition of β-glucans (polysaccharides that occur naturally in the cell walls of bacteria and yeasts) as a supplement to fish diet enhances non-specific immune reactions such as phagocytosis (Dalmo and Bøgwald 2008), resulting in an improved reaction to stress (Jeney et al. 1997; Dawood et al. 2017). While the exact mechanism of this positive effect has yet to be identified, the process is being increasingly studied (Meena et al. 2013) and a number of credible hypotheses exist (Wang et al. 2017).

Vitamins are not usually synthesised in the fish body and need to be administered as feed supplements (Wang et al. 2017). Vitamins C (L-ascorbic acid) and E (α-tocopherol) play an important role in both non-specific and specific immune responses (Anderson 1992; Sahoo and Mukherjee 2002; Misra et al. 2007) though excessive supplementation with vitamin E can be counterproductive, changing it from an antioxidant to a prooxidant. Vitamin C can facilitate regeneration of vitamin E and in this way enhance its effect (Puangkaew et al. 2004; Hamre 2011).

Immunostimulants have been shown to positively affect both innate immunity and general health of fish (Siwicki et al. 1994; Dalmo and Bøgwald 2008; Nya and Austin 2009; Sharifuzzaman and Austin 2009; Giannenas et al. 2015). Administration in combination with a vaccine is also possible, thus providing both specific and non-specific protection. Antibody production in fish, however, is not as advanced as in mammals, leading to difficulties with specific immunisation (Hastings and Ellis 1988; Michel et al. 1990). Activation of the specific immune response also takes longer in fish and requires higher temperatures. As such, non-specific immunity tends to be the main mechanism involved in the infection process (Magnadóttir 2006; Esteban et al. 2015), but significant natural variability needs to be taken into account (Anderson 1992).

In rainbow trout, immunostimulants have been shown to have a positive effect in controlling furunculosis, one of the most common trout diseases (Siwicki et al. 1994; Irianto and Austin 2002; Kim and Austin 2006). In the Czech Republic, this disease is one of the most serious problems in intensive salmonid fish farming systems (Palíková et al. 2014), caused by the Gram-negative bacterium *Aeromonas salmonicida*. High water temperatures (15–21 °C), organic pollutants, high stocking densities and other factors can all facilitate the development and spread of the pathogen.

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and deficient antioxidant defence systems. It affects various biological macromolecules and processes, and may lead to protein damage, lipid peroxidation, genotoxicity and immunotoxicity or inflammation. One of the most important biomarkers involved in cellular defence against overproduction of ROS is the antioxidant glutathione (Hellou et al. 2012). The ratio of the reduced form of glutathione (GSH) and its oxidised form, glutathione disulphide (GSSG), is an important biomarker providing information on the extent of oxidative stress in a biological system. Oxidation of lipids by ROS through the formation of peroxides can be monitored by measuring the end products of the reaction, represented by malondialdehyde (MDA), commonly assessed as a conjugate with thiobarbituric acid (TBARS) (Lushchak 2011). Oxidative stress can be caused by various stressors (Kopp et al. 2018) and can also be used for water quality assessment (Havelková et al. 2009).

The aim of this study was to examine the effects of three commercial diets (a standard diet, a diet supplemented with immunostimulants [β-glucan, vitamins C and E], and a plant-based diet) on oxidative stress and selected immune and haematological indices in rainbow trout. Because the influence of different diets may not manifest itself under normal conditions, an experimental infection with *A. salmonicida* was also conducted.

With immunostimulants, we expected an improvement in the non-specific immune response, a reduction in oxidative stress levels and also a reduction in losses caused by furunculosis. Because plant-based diets are not natural for rainbow trout, we anticipated the opposite effect with this feed. The exact composition of these commercial diets is a trade secret (BioMar, Brande, Denmark); however, knowledge of their effects on fish health is extremely important for fish farmers using these feeds, therefore this study has a great practical impact.

Materials and Methods

Fish and rearing conditions

Rainbow trout (average weight 244.08 ± 43.12 g, total length 26.73 ± 1.35 cm) were kept in an experimental recirculating system consisting of three breeding tanks with a volume of 1000 l (Mendel University in Brno, Czech Republic). Each tank was stocked with 40 fish, except for the standard 920 group (30 fish; see Experimental diets and feeding) and rearing with different diets commenced after the deployment. The water was saturated with oxygen at an average value of 8.17 ± 0.10 $mg \cdot l^{-1}$ (i.e. 85.7 ± 1.1%), average temperature was maintained at 16.6 ± 0.2 °C, pH at 7.76 ± 0.09, NH_3 at 0.001 ± 0.001 $mg \cdot l^{-1}$, $N\text{-}NO_2^-$ at 0.22 ± 0.02 $mg \cdot l^{-1}$ and Cl^- at 70.67 ± 0.35 $mg \cdot l^{-1}$. A Nexus 310 biofilter with a volume of ca. 300 l was used to filter the water; ozone and a UV-C lamp (AquaForte, Doornhoek, Netherlands) were used for disinfection in the first part of the experiment. All applicable international, national, and institutional guidelines for the care and use of animals were followed by the authors. The experiment was performed in accordance with EU Directive 2010/63/EU for animal experiments. The protocol and all procedures employed during this study were ethically reviewed and approved by the Czech Ministry of Education, Youth and Sports.

Experimental diets and feeding

The experimental diets used in this experiment comprised three commercially available feeds (Tables 1 and 2). BioMar EFICO Enviro 920, a standard extruded feed for salmonid fish rearing, was used as a standard (Group "920"). The second feed (Group "Focus"), BioMar EFICO Enviro 920 Focus Plus, was supplemented with immunostimulants; and the third (Group "Advance"), BioMar EFICO Enviro 920 Advance, contained a high percentage of plant components and less fish meal (< 10%). Feeding intensity was based on the supplier's (BioMar, Brande, Denmark) recommendations and ranged from 1.26 to 1.28% of fish weight per day. All fish were fed × 3 a day for 28 days.

Table 1. Experimental diets and their composition.

Code	Commercial name	Significant differences in composition	Pellet size
920	BioMar EFICO Enviro 920	Standard extruded feed for salmonid fish rearing	4.5 mm
Focus	BioMar EFICO Enviro 920 Focus Plus	+ immunostimulants (0.5%) (β-glucan, vitamins C and E)	4.5 mm
Advance	BioMar EFICO Enviro 920 Advance	+ plant components* (> 65%), < 10% fish meal	4.5 mm

*Wheat, soy protein concentrate, sunflower cake, horse bean, wheat gluten, and rapeseed oil
The exact content of each component is a trade secret (BioMar, Brande, Denmark). The basic nutritional composition is shown in Table 2.

Table 2. Experimental diets and their composition (BioMar, Brande, Denmark). Analysis was carried out at Mendel University in Brno (2015).

Feed	Dry matter (%)	Protein (%)	Lipid (%)
BioMar EFICO Enviro 920	95.21	45.88	26.61
BioMar EFICO Enviro 920 Focus Plus	94.76	44.67	26.55
BioMar EFICO Enviro 920 Advance	95.07	43.68	26.99

Experimental infection

After 28 days, the fish were transported in tanks with aeration to the University of Veterinary Sciences Brno (VetUni), Czech Republic. Subsequently, 20 fish from each variant (920, Focus, Advance in duplicates) were infected intraperitoneally with *A. salmonicida* (laboratory strain No. 89407; 2×10^2 colony-forming units per

fish), whereas the remaining fish (control) from each variant were injected intraperitoneally with a sterile saline used as placebo (resulting in the total number of six groups in duplicates). The fish were kept in a recirculating system consisting of six breeding tanks with a volume of 1000 l, each divided into two parts. Conditions during this part of the experiment were the same as in the first part, including feeding.

Sample collection

The fish were observed at the VetUni for 20 days after challenge. After this period, the rearing was terminated, blood was collected from the caudal vein and the fish were humanely euthanized by a blow to the head. Samples of liver, kidney, intestine (washed) and muscle tissue were collected, preserved at -85 °C and later homogenised using a MP Bio homogeniser (MP Biomedicals, Solon, USA).

Markers of oxidative stress

Lipid peroxidation assay using thiobarbituric acid reactive substances (TBARS)

The level of TBARS was measured as a biomarker of lipid peroxidation status using a previously published methodology (Lebedová et al. 2016), with all samples analysed in duplicate.

Assay of glutathione levels

The levels of GSH, GSSG and the GSH/GSSG ratio were simultaneously determined in tissues according to a previously published method (Bláhová et al. 2014).

Determination of the protein content

The content of protein was determined using the commercial Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, USA) based on the Lowry assay (Lowry et al. 1951) and according to the manufacturer's instructions.

Blood indices

The blood indices examined in this study included both haematological (haematocrit level, erythrocyte count, mean corpuscular volume [MCV], mean corpuscular haemoglobin [MCH], mean corpuscular haemoglobin concentration [MCHC], haemoglobin level, leukocyte count and differential leukocyte count) and immunological (respiratory burst, level of specific antibodies, total immunoglobulin [Ig] level and total complement activity) indices.

Part of the blood sample was immediately used to prepare blood smears and to measure haemoglobin, haematocrit and phagocyte respiratory burst value; another portion was cooled and used 3 h later for the assessment of the erythrocyte and leukocyte counts. The third portion was centrifuged immediately (10 min, 1000 *g*) and plasma was stored at -20 °C.

Haematological indices

The assessment of MCV, MCH, MCHC, red and white blood cell counts (RBC, WBC), haematocrit value (PCV) and haemoglobin concentration was done according to previously published methods (Svobodová et al. 2012). Blood smears were stained using May-Grünwald and Giemsa-Romanowski stains and 200 leukocytes were counted for each smear and classified as neutrophilic granulocytes, lymphocytes or monocytes.

Respiratory burst

Luminol-enhanced chemiluminescence was used to assess respiratory burst activity of peripheral blood leukocytes, a measure of phagocyte activity. Phagocytes were activated using opsonised Zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, St. Louis, USA; opsonised by incubation with fish serum) at a final concentration of 0.25 $mg \cdot ml^{-1}$. The luminescent signal produced by luminol dissolved in borate buffer (pH = 9) was then measured using a LM01-T luminometer (Immunotech, Prague, Czech Republic). Fresh blood samples were diluted × 50 in Hank's balanced buffer solution and the results expressed as the maximum intensity (peak in relative light units [RLU]) and total intensity of oxidative burst defined as an integral of the reaction curve area (RLU·s) (for details see Buchtíková et al. 2011). All values were adjusted to 1000 phagocytes.

Specific antibodies

In-house ELISA for the detection of specific antibodies against *A. salmonicida* was prepared analogously to Cinkova et al. (2010).

Total immunoglobulin

Total Ig levels were measured using zinc sulphate (0.7 mM $ZnSO_4 \cdot 7H_2O$; pH 5.8) precipitation according to McEwan et al. (1970). Quantification of IgM concentration was calculated as the total protein level present in the sample before and after precipitation using a commercially available kit (Bio-Rad Laboratories, Hercules, USA). The final Ig concentration (in $mg \cdot l^{-1}$) was determined as the difference between the total protein concentration and the concentration of proteins in the supernatant after precipitation and centrifugation.

Complement activity

Complement activity was measured using the bioluminescent strain of *Escherichia coli* K12 (pEGFPluxABCDEamp) described by Atosuo et al. (2013). Light emission produced by living cells was measured using a LM01-T luminometer (Immunotech, Prague, Czech Republic). Total complement activity was determined against 100 000 cells/well, a diminishing light signal being positively correlated with decreasing bacterial viability. Relative results for complement activity were computed as the difference between the final time of measurement (2 h) and the time needed to kill 50% of bacterial cells.

Statistical analysis

Statistical analysis was carried out individually for each of the variables, with means and standard deviations calculated using the RStudio software (RStudio, PBC, USA). Extreme values revealed by scatterplots (complement with one very low value in the challenged Advance group and five unusually high values in the control Advance group, and a biased sequence of unrealistically low leukocyte counts in ten samples) were excluded. Censored values (four left-censored Ig values) were treated as values lower than a minimal value within the groups (each being replaced by a constant derived from log-transformed distribution as a minimal value of the group minus the difference between the median of the group and the minimal value). Normality was confirmed using the Shapiro-Wilk normality test, with data departing from normal distribution transformed logarithmically. The two-sample *t*-test was performed to reveal significant differences between challenged and control groups and a two-way analysis of variance (ANOVA) was used to evaluate the possible influence of both different nutrition and challenge within the groups. In the case of a significant difference between the diets, the Tukey *post hoc* test (Honestly Significant Difference test) was performed (this test is unable to treat the two-way design). The Kruskal-Wallis test (a non-parametric analogue of the ANOVA method) was applied on the primary (log-transformed) data of the complement parameter to confirm the results. Differences were considered significant at $P < 0.05$.

Results

Mortality

Two deaths occurring six and ten days after challenge were recorded in the Advance group (a mortality rate of 10%). A 5% mortality rate (one fish) was observed in the 920 group seven days after challenge. No mortality was observed in the first part of the experiment (before challenge). Clinical signs observed in the infected fish included inappetence and lethargy. The intensity of these signs corresponded with the mortality rate and was most pronounced in the Advance group, followed by the 920 group.

Oxidative stress

Response of biochemical markers to nutrition

As a first step, the influence of the three diet types (920, Focus, Advance) on oxidative stress indicators in the control (i.e. non-challenged) fish was compared. The highest GSH

Table 3. Biomarker levels in tissues of control groups fed the 920 (standard), Focus (immunostimulants) and Advance (plant-based) experimental diets.

	Liver			Intestine		
	920	Focus	Advance	920	Focus	Advance
GSH*	1361 ± 121 ^{a}	1935 ± 274 ^{b}	2595 ± 333 ^{c}	929 ± 85 ^{ab}	1012 ± 164 ^{a}	1162 ± 80 ^{ac}
GSSG	7.5 ± 2.3 ^{a}	7.9 ± 2.4 ^{a}	4.5 ± 1.6 ^{b}	11.4 ± 3.5 ^{ab}	8.1 ± 3.3 ^{a}	7.1 ± 2.9 ^{ac}
GSH/GSSG	199 ± 61 ^{a}	274 ± 105 ^{a}	617 ± 159 ^{b}	88 ± 27 ^{a}	140 ± 46 ^{b}	195 ± 89 ^{c}
TBARS	0.11 ± 0.03	0.11 ± 0.07	0.10 ± 0.06	13.8 ± 2.3 ^{ab}	19.5 ± 7.8 ^{a}	21.0 ± 5.2 ^{ac}
	Kidney			Muscle		
	920	Focus	Advance	920	Focus	Advance
GSH	1504 ± 226 ^{a}	1777 ± 198 ^{b}	1472 ± 167 ^{a}	276 ± 53	287 ± 79	254 ± 29
GSSG	5.3 ± 1.7 ^{b}	8.8 ± 2.3 ^{c}	3.5 ± 1.4 ^{a}	0.07 ± 0.02 ^{ab}	0.06 ± 0.03 ^{a}	0.04 ± 0.02 ^{ac}
GSH/GSSG	314 ± 135 ^{b}	214 ± 50 ^{a}	482 ± 164 ^{c}	4586 ± 1268 ^{ab}	5717 ± 2869 ^{a}	7834 ± 3382 ^{ac}
TBARS	0.25 ± 0.06	0.24 ± 0.09	0.21 ± 0.04	0.24 ± 0.14	0.23 ± 0.16	0.17 ± 0.04

* GSH = reduced glutathione, GSSG = glutathione disulphide (all nmol·g^{-1} tissue), GSH/GSSG ratio and TBARS = lipid peroxidation (nmol.mg^{-1} proteins)

Values (means ± SD, n = 10) in the same row with different superscript letters are significantly different ($P < 0.05$).

levels were observed in metabolically active liver tissue, whereas the lowest (× 5–10) were found in muscle. Very low GSSG concentrations were recorded in muscle; however, GSSG levels in all the other organs (liver, kidney, intestine) were comparable and the differences less pronounced than with GSH levels. Naturally high TBARS were observed in the intestine, with levels around two orders of magnitude higher than in the other organs (Table 3).

Compared to the 920 diet, the Advance diet significantly increased GSH levels in the liver and intestine, and decreased GSSG levels in the same organs (along with muscle and kidney). The Focus diet, on the other hand, increased GSH concentrations in the liver and kidney, but had no effect on GSSG in the liver and muscle. In contrast to the Advance feed, Focus increased GSSG levels significantly in the kidney. Modulation of TBARS was only recorded in the intestine of the fish fed the Advance diet (1.5-fold increase compared to the 920 diet), with no change noted between the other experimental groups or tissues (Table 3).

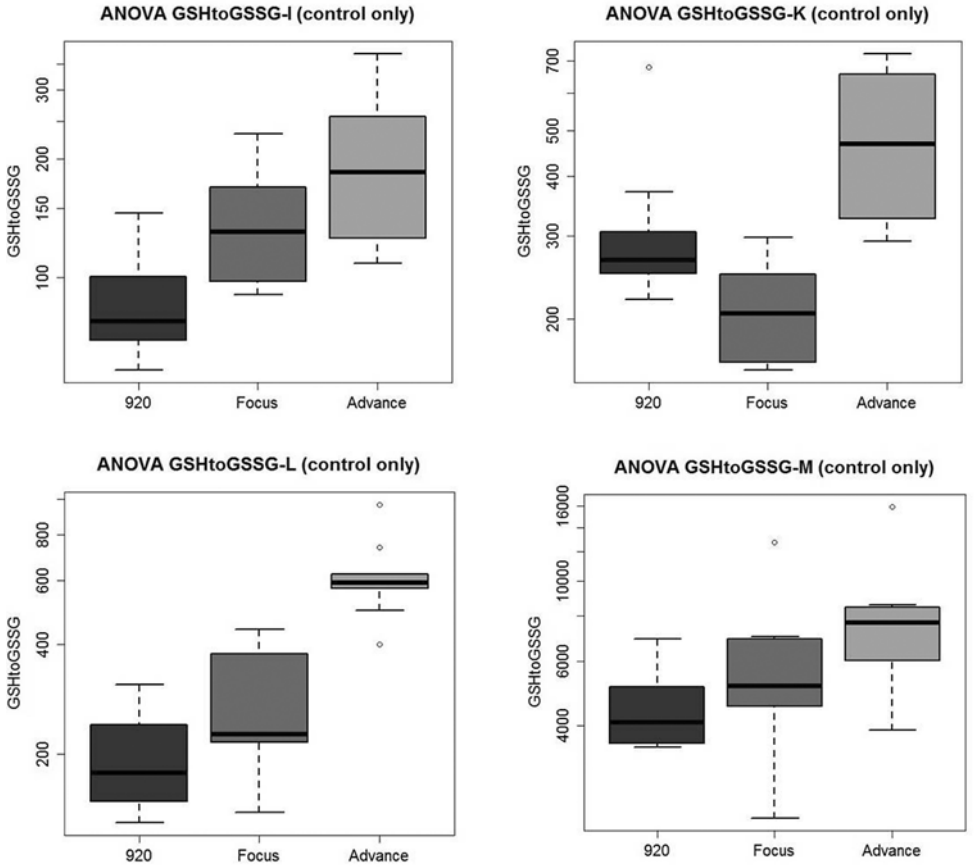

Fig. 1. The ratio of the reduced form of glutathione and its oxidised form, glutathione disulphide in the intestine, kidney, liver and muscle of control groups fed the 920 (standard), Focus (immunostimulants) and Advance (plant-based) experimental diets (ANOVA, n = 10).
Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box). GSH = reduced glutathione, GSSG = glutathione disulphide, I = intestine, K = kidney, L = liver, M = muscle

Systemic responses were observed in the GSH/GSSG ratio, with the greatest effect obtained with the Advance feed, which caused a 1.5- to 3-fold ratio increase in all organs (ANOVA mean values; $P < 0.05$; Fig. 1). On the other hand, the Focus feed had no effect on the GSH/GSSG ratio in the liver and muscle, but significantly increased the ratio in the intestine compared to the 920 feed and decreased it in the kidney in contrast to the Advance feed (Table 3).

In summary, the Advance diet had a clear systemic effect, especially in the intestine where it modulated all biomarkers (Fig. 2).

Response of biochemical markers to challenge

In the fish fed the 920 diet, TBARS were elevated 1.5-fold in the intestine after challenge ($P = 0.046$), accompanied by a 1.5-fold decrease in GSSG levels ($P = 0.03$) and a related 1.5-fold increase in the GSH/GSSG ratio ($P = 0.02$). In the other organs, non-systemic

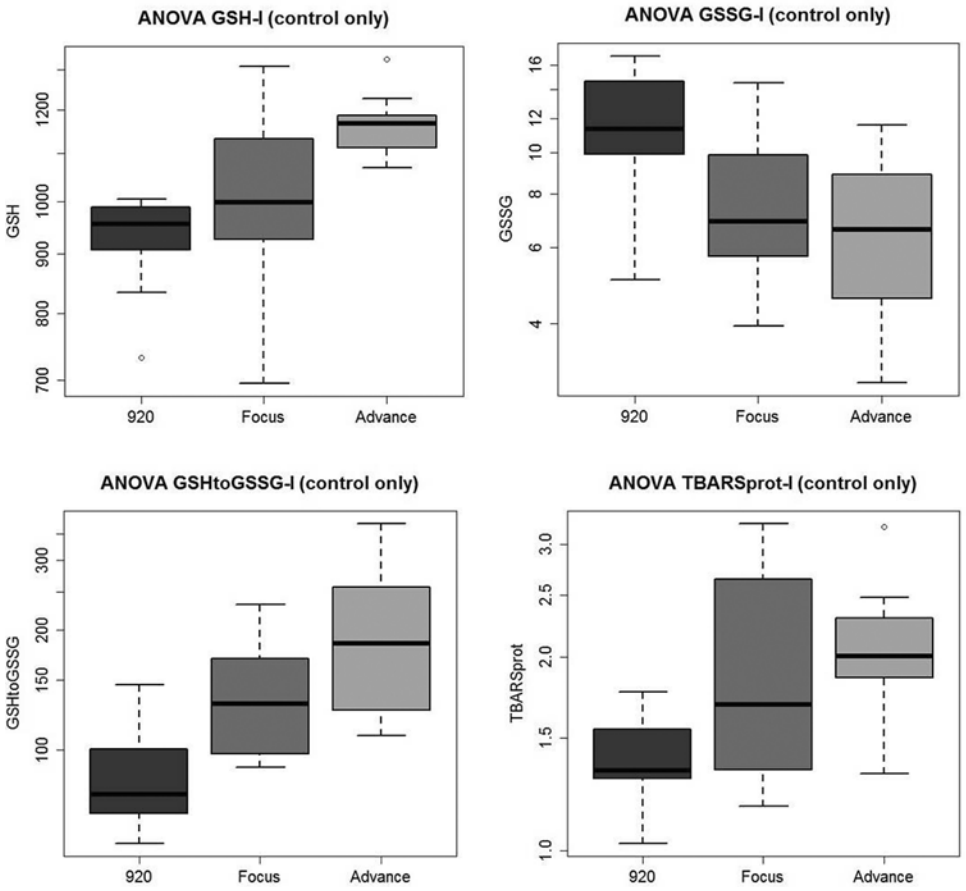

Fig. 2. Reduced and oxidised glutathione (nmol·g^{-1} tissue), their ratio and thiobarbituric acid reactive substances (nmol·mg^{-1} proteins) in the intestine of control groups fed the 920 (standard), Focus (immunostimulants) and Advance (plant-based) experimental diets (ANOVA, n = 10). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).
GSH = reduced glutathione, GSSG = glutathione disulphide, TBARS = thiobarbituric acid reactive substances

differences were recorded, including a 1.3-fold increase in GSH levels in the liver (P = 0.003) and a 1.3-fold increase in the GSH/GSSG ratio in the kidney (P = 0.023). Higher values were always recorded in the challenged groups (Fig. 3).

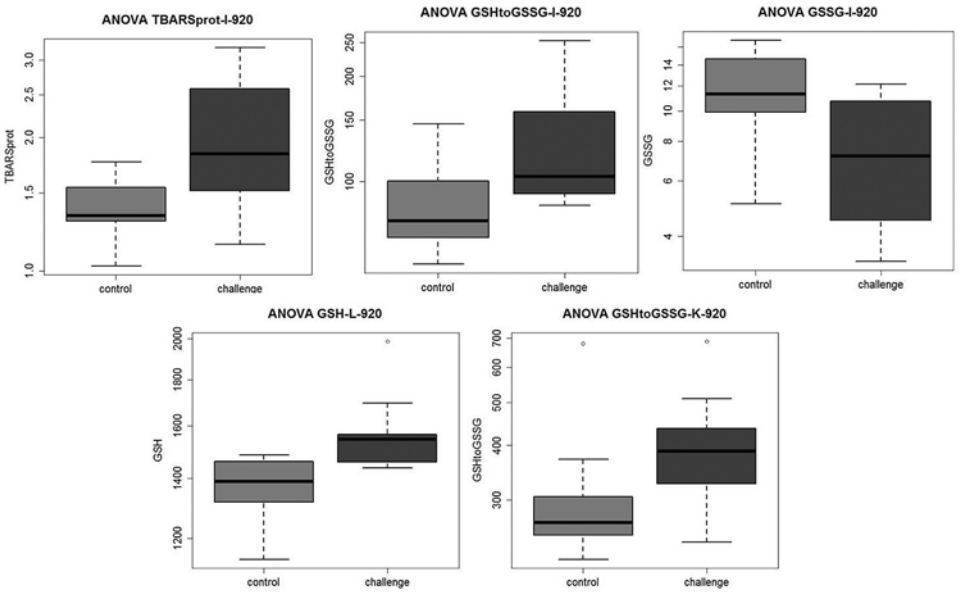

Fig. 3. Thiobarbituric acid reactive substances (nmol·mg^{-1} proteins), the ratio of the reduced form of glutathione and its oxidised form, glutathione disulphide (nmol·g^{-1} tissue) in the intestine, liver and kidney of the 920 (standard) group after challenge (ANOVA, n = 10). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).
TBARS = thiobarbituric acid reactive substances, GSH = reduced glutathione, GSSG = glutathione disulphide, I = intestine, K = kidney, L = liver

While the challenged fish fed the Advance diet had a 1.7-fold increase in TBARS in the kidney (P = 0.0004) and a 1.9-fold increase in muscle (P = 0.02), no difference in GSH, GSSG or their ratio between non-challenged and challenged fish was recorded in any tissue (Fig. 4).

After the challenge, the Focus diet had no effect on GSH levels in any of the studied organs. However, the challenged group displayed a significant decrease in GSSG concentrations in the liver and kidney (1.6- and 2.4-fold, respectively; $P < 0.01$), and a corresponding elevation in the GSH/GSSG ratio in the same organs (1.8-fold in liver and 2.9-fold in kidney; $P < 0.01$). While TBARS showed a 2.3-fold increase in the muscle of challenged fish (P = 0.01), no other organ showed any significant change (Fig. 5).

Protein content

Protein levels (mg per g of fresh weight) showed no significant change in any of the diet groups before or after challenge.

Immunological indices

Response of immunological indices to nutrition

A significant increase (P = 0.006) in the chemiluminescence (CL) area was measured in the Advance group compared to the 920 group. A similar increase ($P < 0.001$) was also

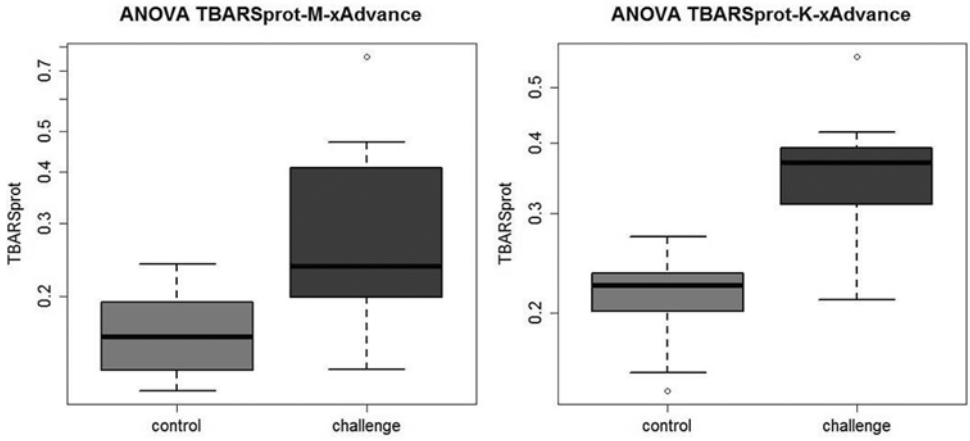

Fig. 4. Thiobarbituric acid reactive substances (nmol·mg^{-1} proteins) in the muscle and kidney of the Advance (plant-based) group after challenge (ANOVA, n = 10). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).
TBARS = thiobarbituric acid reactive substances, M = muscle, K = kidney

observed in all other respiratory burst indicators compared to the other groups (Fig. 6). Compared to the Advance diet, the Focus diet resulted in a significant increase ($P = 0.006$) in total Ig levels (Fig. 7). Complement activity, on the other hand, was significantly higher in the Advance group compared to the 920 ($P = 0.013$) and Focus ($P < 0.001$) groups, and in the 920 group ($P < 0.001$) compared to the Focus group (Fig. 8).

Response of immunological indices to challenge

After challenge, a significantly elevated CL area in the Advance group ($P = 0.008$) and recalculated CL peak in the 920 group ($P = 0.003$) was revealed (Fig. 9). A significant decrease ($P = 0.004$) in specific antibody levels was observed in the 920 group (Fig. 10), whereas total Ig levels showed no significant difference after challenge. Total plasma complement activity showed a significant decrease in the 920 group (Fig. 11).

Haematological indices

Response of haematological indices to nutrition

No significant difference in haematological indices was observed before experimental infection.

Response of haematological indices to challenge

A significant increase of PCV values was recorded in the 920 ($P = 0.030$) and Focus ($P < 0.001$) groups in reaction to the experimental infection (Fig. 12). While a small but significant increase ($P = 0.047$) in the erythrocyte count was recorded in the 920 group, no significant change in MCV, MCH, MCHC or haemoglobin level was observed (Fig. 13).

White blood cell indices showed no significant difference in the total leukocyte or absolute lymphocyte count. In the Focus group, a significant increase ($P = 0.001$) in the percentage lymphocyte count and a significant decrease in the percentage ($P = 0.008$) and absolute ($P = 0.012$) granulocyte counts (and, consequently, in the percentage [$P = 0.007$] and absolute [$P = 0.010$] phagocyte counts) was recorded. Similar, though non-significant changes were observed in the 920 group (Figs 14, 15, 16).

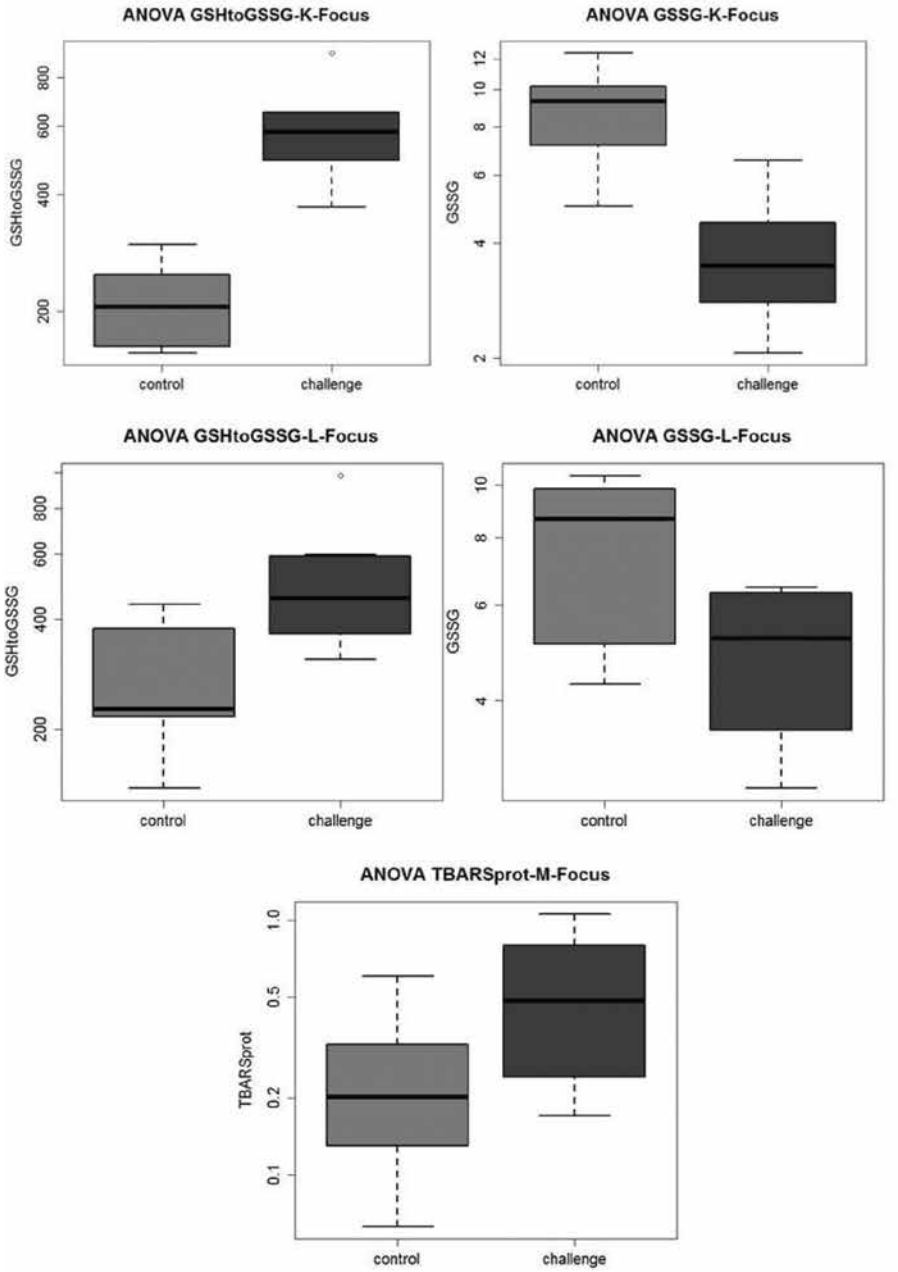

Fig. 5. The ratio of the reduced form of glutathione and its oxidised form, glutathione disulphide (nmol·g^{-1} tissue) and thiobarbituric acid reactive substances (nmol·mg^{-1} proteins) in the kidney, liver and muscle of the Focus (immunostimulants) group after challenge (ANOVA, n = 10). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).
GSH = reduced glutathione, GSSG = glutathione disulphide, TBARS = thiobarbituric acid reactive substances, K = kidney, L = liver, M = muscle

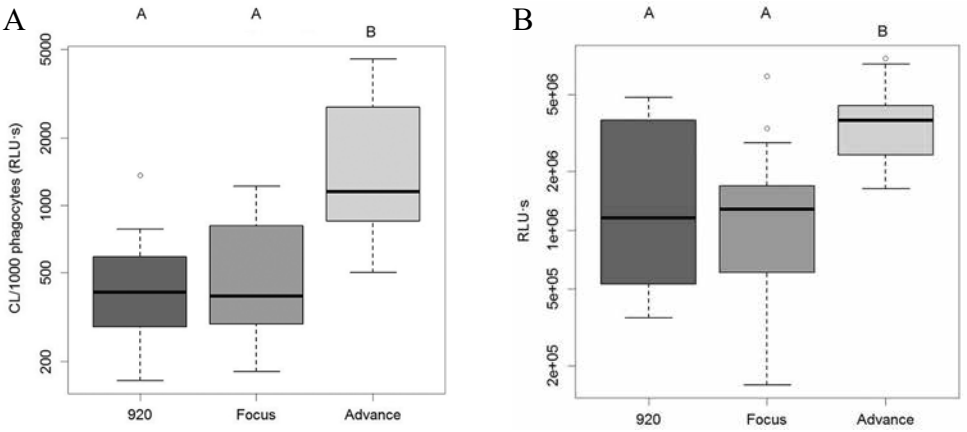

Fig. 6. Respiratory burst in control groups fed the 920 (standard; n = 10), Focus (immunostimulants; n = 20) and Advance (plant-based; n = 20) diets expressed as the integral of chemiluminescence. (A) Total intensity defined as reaction curve area; (B) the same values adjusted to 1000 phagocytes (ANOVA). Values with different letters are significantly different (Tukey *post hoc* test; $P < 0.001$). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).
RLU = relative light units

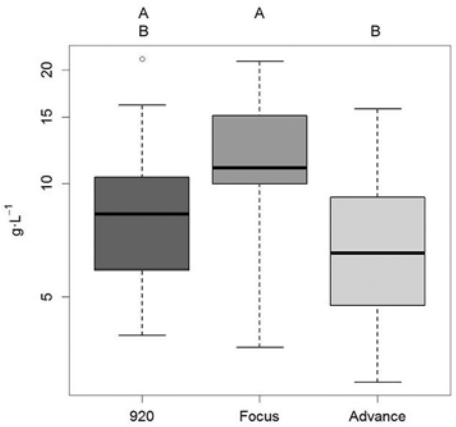

Fig. 7. Total immunoglobulin levels in control groups fed the 920 (standard; n = 10), Focus (immunostimulants; n = 20) and Advance (plant-based; n = 20) diets (ANOVA). Values with different letters are significantly different (Tukey *post hoc* test; $P < 0.006$). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

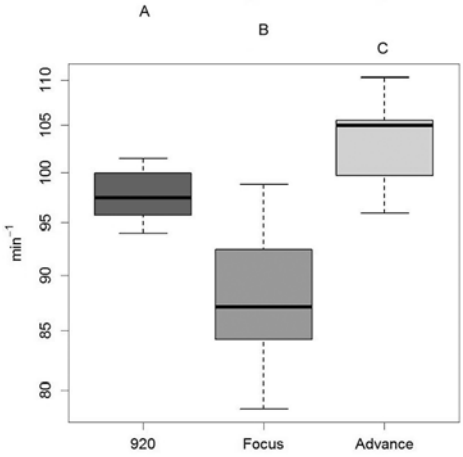

Fig. 8. Complement activity in control groups fed the 920 (standard; n = 10), Focus (immunostimulants; n = 20) and Advance (plant-based; n = 20) diets (ANOVA). Values with different letters are significantly different (Tukey *post hoc* test; 920/Advance, $P = 0.013$; other values $P < 0.001$). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

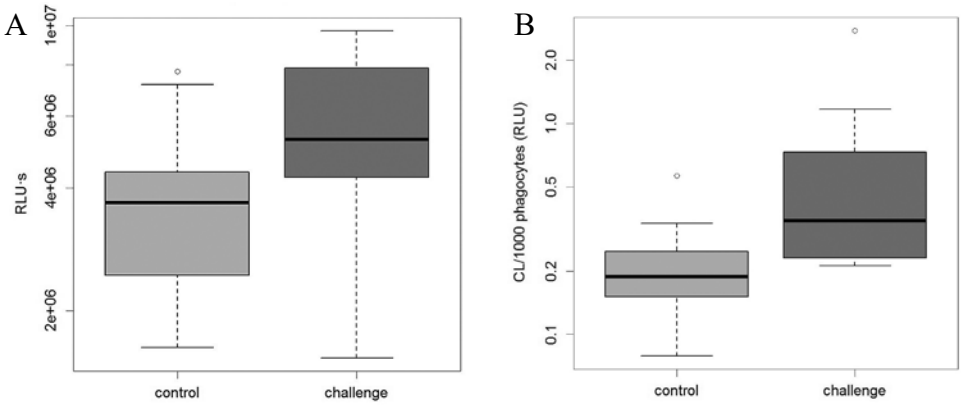

Fig. 9. Chemiluminescence area in the Advance (plant-based) group (n = 20/18; $P = 0.008$ (A) and adjusted chemiluminescence peak in the 920 (standard) group (n = 10/19; $P = 0.003$ (B) after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box). RLU = relative light units

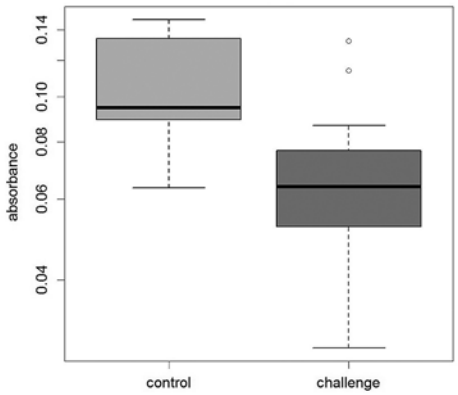

Fig. 10. Specific antibody levels measured as absorbance at 450 nm (OD450) in the 920 (standard) group (n = 10/19; $P = 0.004$) after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

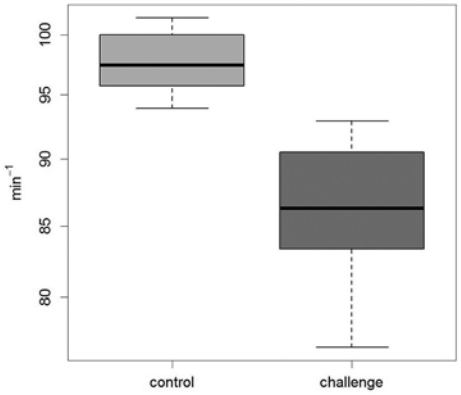

Fig. 11. Complement activity in the 920 (standard) group (n = 10/19; $P < 0.001$) after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

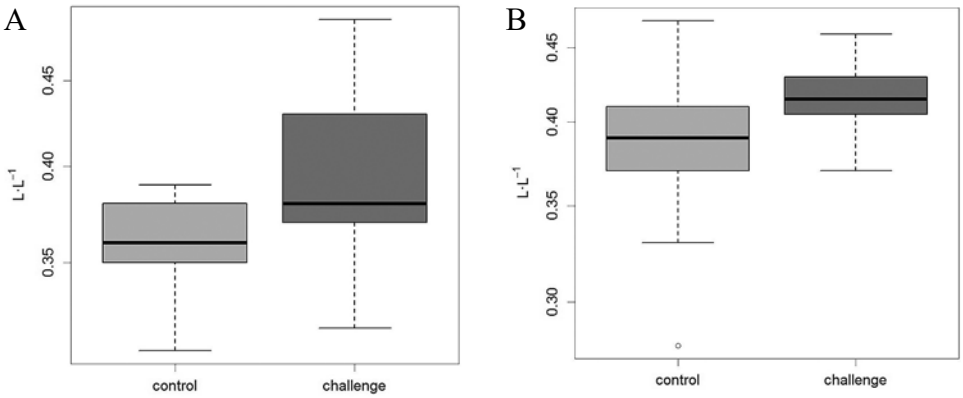

Fig. 12. Haematocrit levels in the (A) 920 (standard; n = 10/19; $P = 0.030$) and (B) Focus (immunostimulants; n = 20/20; $P < 0.001$) groups after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

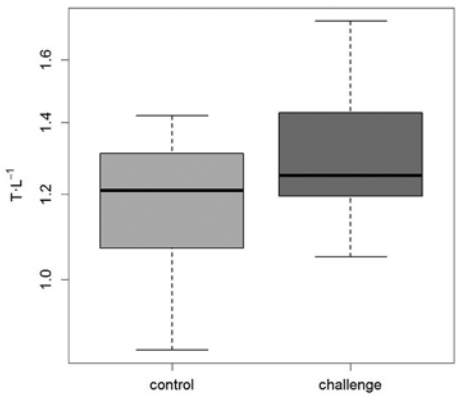

Fig. 13. Erythrocyte count in the 920 group (standard; n = 10/19; $P = 0.047$) after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

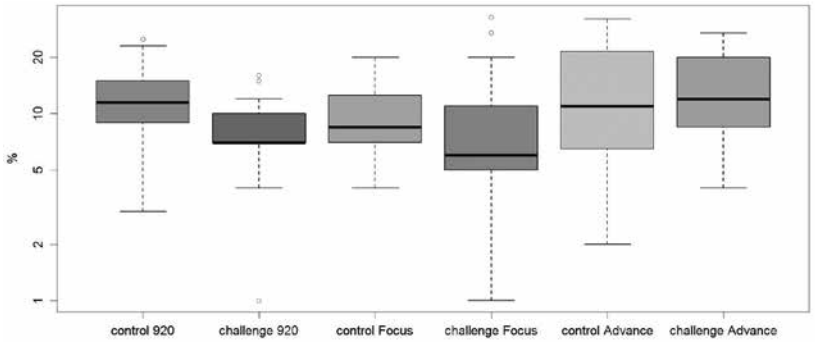

Fig. 14. Percentage phagocyte count in the 920 (standard; n = 10/19), Focus (immunostimulants; n = 20/20; $P = 0.001$ and 0.007) and Advance (plant-based; n = 20/18) groups after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

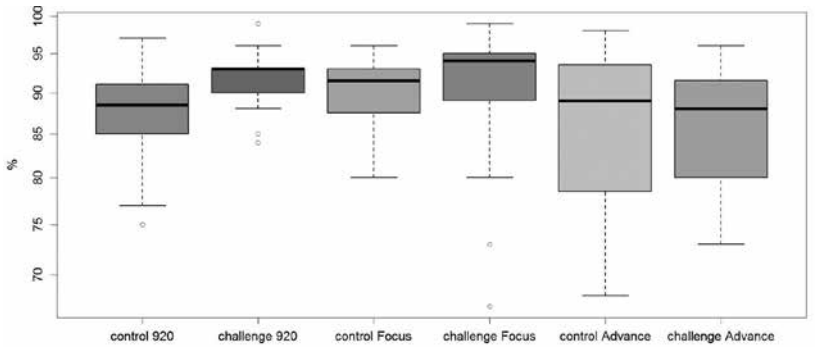

Fig. 15. Percentage lymphocyte count in the 920 (standard; n = 10/19), Focus (immunostimulants; n = 20/20; $P = 0.001$ and 0.007) and Advance (plant-based; n = 20/18) groups after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

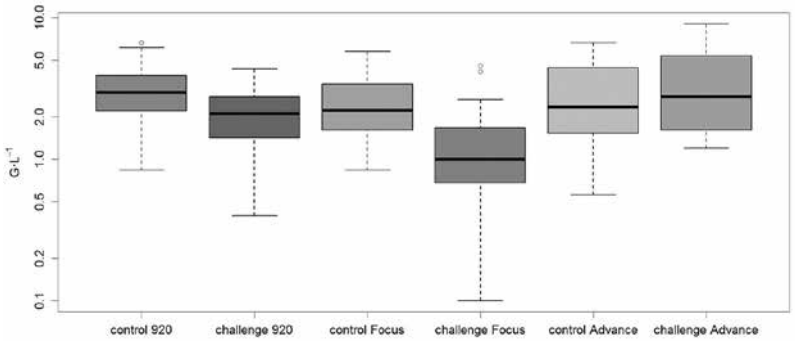

Fig. 16. Absolute phagocyte count in the 920 (standard; n = 10/19), Focus (immunostimulants; n = 20/20; $P = 0.010$) and Advance (plant-based; n = 20/18) groups after challenge (ANOVA). Box plots show the median, the lower and upper quartile representing the 25–75 percentile range, whiskers (upper quartile + 1.5 × IQR and lower quartile - 1.5 × IQR; IQR = interquartile range) and outliers (circles outside the box).

Discussion

An imbalance between the production and removal of ROS (affected also by the immune system) leads to oxidative stress and a range of pathophysiological processes (Koprucu et al. 2015). In addition, the level of unsaturated fatty acids or antioxidants in the diet may also affect redox balance and ROS production. Up until now, distribution of GSH and GSSG in tissues has been investigated in mammals, but a lack of information on their levels in fish tissues still remains present (Hellou et al. 2012). Furthermore, most studies have focused only on GSH levels. In our study, the highest levels of GSH in rainbow trout were found in the liver and kidney, followed by the intestine, with the lowest concentrations found in muscle. Assessment of GSSG showed similar levels in almost all tissues except muscle, where concentrations were around two orders of magnitude lower. Comparable levels and similar distribution of GSH were observed in gilthead seabream (*Sparus aurata*) fed a vegetable oil and carbohydrate rich diet (Castro et al. 2016). Likewise, a similar GSH range in tissues was observed in carnivorous and benthophagous fish species in the field (Sevcikova et al. 2015), cultured juvenile rainbow trout kept without feeding for five days (Stephensen et al. 2002), and Atlantic salmon (*Salmo salar*) fed diets enriched with vitamins, amino acids, and antioxidants (Hamre et al. 2016). Comparable values were also reported in the kidney and liver of rainbow trout fed a diet rich in n-3 polyunsaturated fatty acids (Ozório et al. 2016). In contrast, few available studies reporting on GSSG levels in fish found different concentrations than those observed in this study. Around × 10 higher levels of GSSG have been recorded in rainbow trout liver (measured using spectrophotometry) (Stephensen et al. 2002) and in fish collected from natural reservoirs (GSH and GSSG assessed using electrochemical methods) (Sevcikova et al. 2015). In another study on Atlantic salmon using a commercially available GSH and GSSG analysis kit (Prod. No. GT40, Oxford Biomedical Research, Oxford, UK), the authors reported again an approximately × 10 higher content of GSSG in muscle (compared to the present study) but similar GSSG levels in the liver (Hamre et al. 2016). The highest GSH/GSSG ratio (an indicator of the intracellular redox state [Srikanth et al. 2014]) recorded in our study was observed in the muscle (one order of magnitude higher), followed by the liver, kidney, and intestine.

The level of TBARS (which corresponds with MDA concentration) is a widely used biomarker of lipid peroxidation. In this study, the highest TBARS were observed in the intestine, followed by the muscle and kidney (two orders of magnitude lower), with the lowest levels detected in the liver. This trend was similar for all experimental groups. Similar concentrations of MDA have been reported in the liver of gilthead seabream (Castro et al. 2016), whereas slightly lower values have been observed in the liver and kidney of broodstock rainbow trout (Koprucu et al. 2015). Though other published biomarker levels seem to be consistent, TBARS in fish intestine differed substantially among different studies. For example, mean values observed in gilthead seabream ranged from 23.6 to 28.5 $nmol \cdot g^{-1}$ (Castro et al. 2016), but from 50.9 to 72.4 $nmol \cdot g^{-1}$ in white seabream (*Diplodus sargus*) (Enes et al. 2012). These values are approximately × 10 higher than those observed in the intestine of rainbow trout in the present study.

In general, differences in biomarker levels between different tissues described by various authors can be attributed to inter-species variability in antioxidant defence, differences in the analytical approaches used, and also other indicators such as health status, age or sex.

Regarding nutrition, TBARS in the liver, kidney and muscle were unaffected by the Advance (plant-based) or Focus (immunostimulants) diets, indicating no significant oxidative stress (lipid peroxidation) in these tissues. The most sensitive tissue, where all oxidative stress biomarkers (GSH, GSSG, GSH/GSSG ratio and TBARS) were significantly modulated, was intestinal tissue of the Advance control group (when

compared to the 920 control feed). In previous studies, neither white seabream fed a non-starch polysaccharide supplemented diet (Enes et al. 2012) nor European sea bass (*Dicentrarchus labrax)* fed a taurine-enriched diet (Feidantsis et al. 2014) showed any modulation of MDA concentration in the intestine. A study on juvenile Malabar grouper (*Epinephelus malabaricuss*) fed a folic acid deficient diet showed enhanced TBARS levels in the liver (Lin et al. 2011), whereas increased MDA levels in the muscle were reported in juvenile largemouth bass (*Micropterus salmoides*) fed a diet rich in oxidised lipids (Chen et al. 2012). The opposite trend (i.e. a significantly improved lipid oxidative stability measured as MDA content) was observed in the muscle of rainbow trout fed a diet rich in essential oil components carvacrol and thymol (Giannenas et al. 2012), and in rainbow trout fed probiotics (Giannenas et al. 2015). The protective role of nutrition on the liver and kidney was also observed in another study with rainbow trout fed a diet supplemented with n-3 polyunsaturated fatty acids (Koprucu et al. 2015). Some previously reported studies with rainbow trout, Atlantic salmon, gilthead seabream and European sea bass have also shown a reduction in oxidative damage linked with plant-based diets containing components with strong antioxidant activity, such as flavonoids, α-tocopherol, vitamin C or astaxanthin (López-Bote et al. 2001; Sitjà-Bobadilla et al. 2005; Olsvik et al. 2011).

With the Advance feed, a significant elevation in intracellular redox state (GSH/GSSG ratio) in all organs (i.e. liver, kidney, muscle and intestine) was recorded. In comparison, the Focus feed caused a significantly higher GSH/GSSG ratio in the intestine only, and, in contrast, a significant decrease in the kidney. To our knowledge, relatively few studies focusing on levels of both GSH and GSSG during feeding experiments have been published. As an example, only minor changes in redox regulation were reported in the liver and muscle of Atlantic salmon fed a diet with high inclusion of plant ingredients, micronutrients, and amino acids (Hamre et al. 2016).

In this study, the data suggest that the Advance feed may up-regulate enzymes involved in the synthesis of GSH, an important antioxidant and detoxification enzyme cofactor (Stephensen et al. 2002; Rymuszka 2012). This assumption is supported by the observed systemic increase in GSH levels in all tissues, accompanied by decreased GSSG. The Focus feed also seems to affect enzyme activity involved in the maintenance of glutathione levels, but only in the intestine.

Besides the influence of nutrition on intact fish, we also investigated biomarker changes in response to challenge by *A. salmonicida* over a 20-day experiment. In fish fed the 920 (standard) diet, TBARS were elevated in the intestine. The presence of *A. salmonicida* in fish intestine has also been reported in previous studies (Hiney et al. 1994; Jutfelt et al. 2006; Fečkaninová et al. 2017). A significantly higher TBARS level was also observed in the kidney and muscle of fish fed the Advance feed, whereas with the Focus Feed, an elevation of TBARS was observed in muscle only. Challenge had no effect on TBARS in the liver of any of the three diet groups compared. With respect to the GSH/GSSG ratio, challenge had no effect on the Advance group; however, non-systemic modulations were observed in fish from the other two diet groups (Focus and 920). We also observed a significant increase in the antioxidant potential in the kidney and an enhanced GSH/GSSG ratio in the liver of the Focus group. To our knowledge, only one comparable study has so far reported any significant increase in oxidative stress in the liver and gills of rainbow trout vaccinated against furunculosis (Tkachenko et al. 2014).

Immunological and haematological indices are frequently used for the assessment of fish health. One of these indices, respiratory burst, is an important defence mechanism against bacterial pathogens (Secombes and Fletcher 1992). During this process, phagocytes, such as neutrophils and monocytes/macrophages, generate ROS using a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase as a mediator (Neumann

et al. 2001; Vera-Jimenez et al. 2013). Previous studies have shown an elevation in respiratory burst after immunostimulant administration (Jeney et al. 1997), though no significant differences were observed in other studies (Ortuño et al. 2000). In a study using probiotics (Sharifuzzaman and Austin 2009), respiratory burst did not differ significantly from the control group after challenge, but did remain enhanced compared to the control throughout the experiment. In our study, administration of the plant-based (Advance) feed resulted in an enhancement of respiratory burst, even in non-challenged fish. Immunostimulants (Focus), on the other hand, showed no significant difference compared to the standard (920). Activation of phagocytes is frequently observed during infection (Palikova et al. 2017), hence the positive effect of the increase in the Advance group before challenge is questionable, and may even be negative in the long-term.

Levels of specific antibodies in the 920 feed showed a significant decrease after challenge, probably related to consumption of antibodies during the infection process, even though no antibodies should be present in the control groups. With the two other feeds, on the other hand, no significant changes were observed. Thompson et al. (1993) used immunisation with *A. salmonicida* and described significantly higher titres of specific antibodies in control fish compared to fish subjected to confinement stress; however, this effect was observable 43 days post-immunisation and not earlier (29 days). Other studies (Verlhac et al. 1998; Irianto and Austin 2002) have also provided support for the hypothesis that more than 20 days may be required for sufficient antibody production. An elevation of specific antibodies has been recorded with glucan (and vitamin C) supplemented diets (Verlhac et al. 1996); however, the positive effect disappeared after 12 weeks. In this study, the total Ig level was significantly elevated in the Focus group. Because vitamin E is probably involved in production of Ig (Puangkaew et al. 2004), it could have positively influenced its values. Such an increase in total Ig level with vitamin C and E supplemented diets has been previously observed (Khara et al. 2016). The benefits of using a multi-strain probiotic as a dietary supplement was demonstrated by Giannenas et al. (2015), with an increase in total complement activity observed (though no bacterial challenge was conducted in this research). A decrease in complement activity has been observed with vitamin E deficient diets (Pearce et al. 2003). In our case, complement activity was significantly higher with the Advance feed (compared to the other feeds) and with the 920 feed (compared to the Focus feed). After experimental infection, however, a significant decrease occurred only in the 920 group, resulting in similar values for the 920 and Focus groups. As with respiratory burst, the highest values were consequently recorded in the Advance group.

Haematological indices showed no significant difference between the control groups; only after challenge did differences become apparent. Haematocrit increased in both the 920 and (even more) in the Focus group. An increase in the erythrocyte count in the 920 challenged group was at the edge of significance. With probiotics, Irianto and Austin (2002) described an increase in the number of erythrocytes and leukocytes after challenge, whereas Kim and Austin (2006) recorded no such findings. A decrease in haemoglobin, haematocrit level and erythrocyte count was observed with vitamin C and E deficient diets (Taveekijakarn et al. 1996; Adham et al. 2000). This observation is also supported by the study of Khara et al. (2016), who described an elevation in these indicators after feeding diets supplemented with the same vitamins. In some cases, a decrease in the erythrocyte count can occur as an undesired side-effect of immunostimulant administration (Vallejos-Vidal et al. 2016). On the other hand, spleen contractions are during stress also capable of increasing PCV (Gallaugher et al. 1992), with an elevation in the circulating erythrocyte count and their swelling often observed (Wendelaar Bonga 1997). According to Svobodova et al. (1994), stress can cause an increase in haematocrit level, erythrocyte count, and haemoglobin concentration. During our study, however, no significant change

in MCV, MCH, MCHC or haemoglobin level was observed. Consequently, the Advance group was the only one displaying no difference in RBC indices after challenge.

No significant differences in the total leukocyte or absolute lymphocyte counts were recorded in the WBC indices. In the Focus group, a significant increase in the percentage lymphocyte count and a decrease in the percentage and absolute granulocyte counts (and consequently, the phagocyte count) occurred after challenge. Similar but non-significant changes were also observed in the 920 group. The reduction in phagocyte numbers in the 920 and Focus groups corresponds with the results of respiratory burst, with the Advance feed providing the highest values. The observed decrease in the percentage lymphocyte count and increase in the phagocyte count could have been caused by stress (Wendelaar Bonga 1997); as such, observation of the opposite findings could be a sign of improved coping mechanisms. According to Engelsma et al. (2003), redistribution and cortisol-induced apoptosis of lymphocytes are the main causes of lymphocytopenia during stress. With immunostimulants, an increase in the percentage lymphocyte count has been observed in several studies (Thompson et al. 1993; Yarahmadi et al. 2016). Similar results (an elevation of the previously stress-reduced percentage lymphocyte number) have been observed after the use of feed containing glucan (Jeney et al. 1997).

To summarise, the Advance (plant-based) feed resulted in significant enhancement of the immune system, even in non-challenged fish. This enhancement may not be positive in the long-term, however, and may be a sign of stress (Tort 2011). Intestinal tissue was the one most sensitive to oxidative stress, with all biomarkers affected in the Advance group. The Advance feed also increased the GSH/GSSG ratio in all tissues (liver, intestine, kidney and muscle), while only slight differences (especially in the intestine and kidney) were recorded with the Focus (immunostimulants) feed. Considering that plant-based nutrition is unnatural for rainbow trout, we anticipated a negative effect on the immune system, whereas immunostimulants were expected to have a positive effect (Siwicki et al. 1994; Dalmo and Bøgwald 2008; Nya and Austin 2009). In our study, the results with the Focus feed were comparable to the standard (920) feed with less distinctive changes after challenge – the stress response seemed to be reduced. The levels of TBARS were significantly elevated only in muscle, whereas the Advance group also showed an increase in the kidney. No elevation in this variable was observed in the intestine, indicating a positive influence on this tissue. Finally, fish fed the Focus feed had the lowest mortality rate (the highest being observed with the Advance feed). Nevertheless, more time may be needed to provide a better understanding of the influence of these diets on fish health (Thompson et al. 1993; Verlhac et al. 1998; Ghaedi et al. 2015).

Based on our findings, we believe that the influence of these commercial feeds, specifically plant-based and immunostimulant-enhanced diet, is not negligible. Significant differences in oxidative stress levels, along with a range of immune and haematological indices, were detected between the diet groups and challenged/control groups. The results of this study have a practical impact for fish farmers using these feeds.

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