The effect of dietary addition of a synbiotic after long-term starvation on certain biochemical parameters and liver structure in common carp (*Cyprinus carpio* L.)

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ABSTRACT

The prolonged starvation has many effects on the physiological and morphological processes in fish and interferes them with the ability to absorb nutrients and caloric intake. The aim of present study was to investigate the effect of long-term starvation and posterior dietary administration of commercial synbiotic consisting fructoligosaccharide (FOS) which used in combination with *Enterococcus*, *Lactobacillus*, *Bifidobacterium* and *Lactococcus* spp. for evaluation on some blood biochemical parameters and histological characteristics of common carp liver. Experimental fish after 5 months starvation period were subjected to the following feeding protocols: continuous starvation until 60 day (ST), re-feeding (FS) with dry composed diet (Aqua 1032 Swim) and refeeding with composed diet and synbiotic (FSS) for the following 60 days. Blood samples were used for analysis of the following plasma parameters: total protein, albumin, globulin, urea, creatinine, cholesterol, AST, ALT, glucose and serum for thyroid hormones. Histology analyses were performed on the carp liver samples. During the experimental period, fasting and refeeding were influenced significant on studied biochemical parameters, except glucose and urea. Also, the statistically largest ($P < 0.001$) difference was observed in albumin and cholesterol values after refeeding. Our results reveal the changes in values of biochemical parameters during long-term starvation and refeeding with synbiotic and indicate signs of structural alterations of the liver in common carp.

Keywords: biochemical parameters, common carp, histological changes, synbiotic

INTRODUCTION

Starvation is a situation that could have happened to many species of fish in native and captive condition in response to some factors – migration, temperature changes, reproductive cycles, weather conditions, etc (Yarmohammadi et al., 2015). Essentially, the negative effect of them is shown through changes on biochemical indices and damages in structure of organs in fish digestive system (Urku et al., 2020). On the other hand, re-feeding can cause different reactions in the body, depending on the period of food deprivation, environmental factors, animal species, etc (Pérez-Jiménez et al. 2007). As a result, in order to reducing the harmful effects, stimulate the digestive process and help restore the natural balance of bacteria in digestive system in animals and humans, there is a growing interest in the use of beneficial bacteria (probiotics) (Syahidah et al., 2015). It is believed that probiotics have a favorable effect on the digestive processes of aquatic animals, because probiotic strains can synthesize extracellular enzymes such as proteases, amylases and lipases, as well as provide growth factors such as vitamins, fatty acids and amino acids (Rinkinen et al. 2003). More attention is paid to the selection of probiotic strains relative to the optimal temperature range for every kind of fish, since they are essential and ensure the possibility of bacterial growth in the digestive system of the host, as well as in the aqueous medium (Vine et al., 2004). Among the substances capable of improving digestion while enhancing the effect of beneficial bacteria in the gastrointestinal tract, prebiotics have been described. Prebiotics are indigestible fiber act by selectively stimulating the growth of *Lactobacillus* and *Bifidobacterium*, which on the host like improving digestion process (Akhter et al., 2015). Lactic acid-producing bacteria, e.g. *Bacillus* and *Enterococcus faecium* are the focus of increased interest in aquaculture sector (Panigrahi et al., 2004; Wang et al., 2000). The most commonly used prebiotics in aquaculture nutrition are inulin, mannanoligosaccharides (MOS) and fructooligosaccharides (FOS). Generally, beneficial effects from application of prebiotics are increase the density and height of intestinal villi and the integrity of the intestinal mucosa (Ringø et al., 2010). Synbiotics refer to dietary supplements combining prebiotics and probiotics in a form of synergism, therefore synbiotics, enhancing their isolated beneficial effects (Cerezuela et al., 2011).

The aim of the study was to investigate the effect of long-term starvation (5 months) and posterior dietary administration of commercial synbiotic consisting fructooligosaccharide (FOS) which used in combination with *Enterococcus*, *Lactobacillus*, *Bifidobacterium* and *Lactococcus* spp. for evaluation on some blood biochemical parameters and histological characteristics of common carp liver.

MATERIALS AND METHODS

Animal and treatment

The 5 months starved common carp (*Cyprinus carpio*) were taken from fish farm "Tundzha 73", Bulgaria and transported in a conveying tank, reinforced with oxygen to the Experimental Base at Trakia University. The 60 fish were randomly assigned in RAS. Experimental fish were subjected to the following feeding protocols: (ST) - continuous starvation until 60 day, (FS) - re-feeding with dry composed diet (Aqua 1032 Swim, Aqua-Garant®, Garant Tiernahrung GmbH) and (FSS) - refeeding with composed diet and 2% synbiotic mix added (OmniBiotic 6, Institut Allergosan Deutschland GmbH) for the following 60 days. Synbiotic (probiotic supplements containing prebiotic components) contained *Lactobacillus casei* W56, *Bifidobacterium animalis* W53, *Lactococcus lactis* W58, *Enterococcus faecium* W54, *Lactobacillus acidophilus* W55, *Lactobacillus salivarius* W57 and fructooligosaccharides (FOS).

Sampling and analyses

Blood samples were drawn from the *v. caudalis* in containers with heparin, centrifuged at 2500 rpm for 15 min (Ohaus FC5515, Ohaus Corp.) and immediately separated and stored at -20°C until analysis. Blood plasma biochemical parameters were analyzed using a semi-auto biochemical analyser (Mindray BA-88, China) with commercial diagnostic kits (Giese, Diagnostics, Italy).

Total triiodothyronine and total thyroxine analyses were conducted with Elisa analyser, HumaReader HS (Human GmbH, Wiesbaden, Germany) and its measurement Elisa kit (Monobind, Inc., USA).

Fish were sacrificed by decapitation according to the Guidelines of the European Union (2010/63/UE) and Animal Welfare Act and approved by the Committee on Animal Experimentation at the Trakia University, Bulgaria. The liver was collected and prepared for histopathological analysis. Samples for histopathology examination were fixed in Davidson's solution at room temperature for 24 h, dehydrated in 70% ethanol, treated with xylene and embedded in paraffin wax. Paraffin blocks were sectioned (4-5 µm thickness) on a microtome Leica RM 2125 (Leica Microsystems GmbH, Austria), dewaxed and stained with haematoxylin (Sigma-Aldrich-HHS16), and eosin (Merck 109844, Merck KGaA, Germany), according to the method described by Culling (1963).

Statistical analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA). The results were processed with software Statistica v.10 (StatSoft Inc., 2002). All results are presented as mean and standard deviation of the

mean (Mean $\pm$ SD). The statistical significance of parameters was determined in the LSD test at $P < 0.05$.

RESULTS AND DISCUSSION

Biochemical parameters

The present work was conducted to evaluate the effects of synbiotic, when administered in feed on certain blood parameters and the possible histological changes that will occur in the liver of common carp after long-term starvation. The results of the biochemical study are presented in table 1.

Table 1. Blood plasma biochemical parameters of carps after long-term starvation and subsequent refeeding

Parameters	ST	FS	FSS
Total protein, g/L	24.33 $\pm$ 6.02	48.6 $\pm$ 14.7**	44.50 $\pm$ 8.73**
Albumin, g/L	2.33 $\pm$ 1.03	9.50 $\pm$ 3.44***	8.66 $\pm$ 2.42***
Globulin, g/L	22.00 $\pm$ 5.17	39.16 $\pm$ 12.48**	35.83 $\pm$ 6.4*
Urea, mM/L	1.42 $\pm$ 0.83	1.53 $\pm$ 0.36	1.42 $\pm$ 0.46
Creatinine, μ mol/L	31.76 $\pm$ 8.2	42.63 $\pm$ 7.55*	42.83 $\pm$ 7.86*
Total cholesterol, mM/L	1.14 $\pm$ 0.17	3.20 $\pm$ 0.78***	3.07 $\pm$ 0.73***
AST, U/L	99.00 $\pm$ 31.81	154.6 $\pm$ 32.3*	162.5 $\pm$ 50.43*
ALT, U/L	25.50 $\pm$ 5.43	38.00 $\pm$ 5.21**	36.5 $\pm$ 7.76**
Glucose, mM/L	2.76 $\pm$ 0.55	2.76 $\pm$ 0.61	2.20 $\pm$ 0.48
Calcium, mM/L	0.75 $\pm$ 0.16	1.24 $\pm$ 0.44*	1.31 $\pm$ 0.33*
Phosphorus, mM/L	1.38 $\pm$ 0.32	1.76 $\pm$ 0.23	2.36 $\pm$ 0.86*
Triiodothyronine, ng/ml	1.97 $\pm$ 0.18	2.69 $\pm$ 0.21	3.06 $\pm$ 0.32
Thyroxine, mcg/dl	1.34 $\pm$ 0.19	1.89 $\pm$ 0.24	2.09 $\pm$ 0.57
T3/T4	1.47	1.42	1.46

Note: Different superscripts *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) indicate significant differences.

During the experimental period, fasting and refeeding were influenced significant on studied biochemical parameters, except glucose and urea.

Changes in the concentration of protein, albumin and globulins are considered as indicators of fish stress (Sala-Rabanal et al., 2003). As a result of starvation, there is a decrease in the levels of protein, albumin and globulins. Low levels of albumin after starvation may be is a consequence of reduced albumin synthesis in hepatocytes due to preferential synthesis in the liver of other proteins, which are important components of the body's defense mechanisms (Gruys et al., 1998). In present investigation, changes in the concentrations of total serum protein, albumin and globulins after refeeding were influenced by the diet. After the start of feeding with a standard diet and diet with synbiotic, the concentration of total protein statistically increase ($P <$

0.01) (48.6 ± 14.7 U/L and 44.5 ± 8.73 U/L, respectively) compared to the ST group (24.33 ± 6.02 g/L). The albumin values in FS and FSS groups show a steady upward trend and reach significant ($P < 0.001$) enhancement (9.5 ± 3.44 g/L and 8.66 ± 2.42) compared to the values recorded the ST group (2.33 ± 1.03 g/L). Likewise, globulins levels reached statistically significant peak elevation ($P < 0.01$) in the FS and FSS experimental groups, respectively 39.16 ± 12.48 g/L and 35.83 ± 6.4 g/L, compared to starved fish (22 ± 5.17 g/L). Previous research has also found that concentration of total protein, albumin and globulin increased after probiotic/synbiotic intake of common carp (Elekary et al., 2014) and trout (Mehrabi et al., 2012).

Fish kidneys contribute little to the excretion of nitrogenous waste, and interpretation of urea nitrogen and creatinine values may not be essential in assessing renal function (Thrall et al., 2012). In current investigation the urea level for all groups were unchanged. Creatinine concentrations did not show a big variation after long-term fasting and subsequent feed intake but was lower in ST group than FS and FSS groups. This finding was earlier confirmed in starved fish by Kulkarni and Barad (2015).

According to results from previous investigations starvation may lead to reduction in cholesterol values (Friedrich and Stepanowska, 2001; Pérez-Jiménez et al., 2012; Eslamloo et al., 2016). During starvation a decrease in total cholesterol level was observed (1.14 ± 0.17 mM/L) than in FS group (3.20 ± 0.78 mM/L) and FSS group (3.07 ± 0.73 mM/L).

Measuring of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in the blood is one of the valuable diagnostic tools (Shahsavani et al., 2010). The effect of starvation on the activity of these two enzymes in fish is variable. In current study the activity of AST and ALT showed statistically significant differences after re-feeding, from 99 ± 31.81 U/L in the ST group to 154.6 ± 32.3 U/L in FS group and 162.5 ± 50.43 U/L in the FSS group. The level of ALT from 25.5 ± 5.43 U/L to 38 ± 5.21 and 36.5 ± 7.76 U/L, respectively. The increasing activities of aminotransferases could be due to raising protein synthesis (Friedrich and Stepanowska, 2001). Similar differences in the action of these enzymes have been described in starved marine fish species, as olive flounder, European sea bass, Senegalese sole, red porgy (Kim et al. 2014).

The system of emergency in aquatic organism to some stressors is illustrated by provocation of the hypothalamus, which leads to activation of the neuroendocrine system, changes in metabolism that allow the body to cope with changing living conditions (Martínez-Porchas et al., 2009). In some cases, the effect of stressors on glucose may increase or decrease concentration it. Some authors suggest that after long-term starvation lowering glucose levels may be due to depletion of hepatopancreatic function and muscle glycogen (Namrata et al., 2011; Shimeno et al., 1997). A similar significant reduction in glucose was observed in carp after fasting for 1 month

(Namrata et al., 2011; Pottinger, 1998), 3 months (Friedrich and Stepanowska, 2001) and 7 months (Atanasoff et al., 2018). In present study, after 7 months of fasting, a glucose value (2.76 ± 0.55 mM/L) was remained unchanged after refed the animals with the standard feed, while with the addition of synbiotic, the levels decreased slightly but not statistically (2.20 ± 0.48 mM/L). Mehrabi et al., (2012) reported small variation in blood glucose levels after dietary use of a commercial synbiotic in rainbow trout.

In groups FSS was observed significant rise of calcium and phosphorus levels compared to the ST and FS groups. The rise of calcium absorption by prebiotic supplements may be due to increase the short chain fatty acids (SCFA) which influence the epithelium to increase calcium absorption or enhance acidity in the large intestine. As a subsequently the lowered colonic pH contributes to the improvement of calcium solubility (Trinidad et al., 1996). Similar findings were also observed after dietary intake of synbiotics containing *Lactobacillus* and *Bifidobacterium lactis* which results in enhance calcium levels (Naughton et al. 2011).

The review of the available literature shows little data on the effect of synbiotics on thyroid hormones in fish. It has been proposed that starvation mainly influences the HPT axis as a reducing conversion of T4 to T3 (Leatherland and Farbridge, 1992; Atanasoff et al., 2021). The results for ST group triiodothyronine and thyroxine in the present study were similar to findings observed in study by Arslan et al. (2015) conducted with starved juvenile carp, where the concentrations also decreased. Taking into account that the control fish were subjected to prolonged starvation, rising T3 in refeeding groups may be the result of supplement. Similar results of T3 and T4 increase after probiotic treatment in aquaculture have been reported by other studies as well Adel et al. (2016), Sun et al. (2021).

Histological Analysis

Histological changes observed in the liver of common carp after prolonged starvation and refeeding is illustrated in Figure 1a,b,c.

Figure 1. Liver section of starved carp (a), FS group carp (b) and FSS group carp (c) (HE x40)

At the end of 7 months' starvation, histopathological alterations were detected in the liver cells in the ST group (hepatic steatosis), whereas the examined sections in FS and FSS groups revealed normal structure. In starved carp hepatocytes had volume shrunk, lipid vacuoles found in the cytoplasm of hepatocytes were reduced in volume and number, compared with the re-feeding groups. The nuclei were smaller and intensively stained (karyopicosis), which a sign of malnourishment (Ostaszewska et al., 2006). The similar histopathological findings were also observed in the trout liver (Liu et al., 2018) and in previous 5 months' study in carp (Urku et al., 2020). In FSS group carp, the boundaries between hepatocytes were clearer. The cell volume was restored and nuclei were deviated to one side. One reason for hepatocyte vacuulations could be the more lipids and glycogen deposition, due beneficial effect of synbiotic on the synthesizing extracellular enzymes and provide nutrient (El-Nobi et al., 2021).

CONCLUSION

The current study demonstrates that the two-month supplementation with synbiotic did not have a significant effect than standard refeeding on assessed parameters. In addition, the results of present work revealed that subsequent refeeding and supplementation of synbiotic have a similar effect in liver structure in carp. Therefore, for a more correct conclusion, further studies with a longer follow-up period and using different doses of synbiotic in starved fish are need.

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