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Research Article

Effects of Grobiotic®-A on growth, whole body composition, and intestinal histology of endangered brown trout *(Salmo trutta macrostigma)*

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ABSTRACT

Brown trout is a very important salmonid species with high economic value and an important aquaculture potential. Due to various reasons, it is in danger of extinction in its natural environment. For this reason, both stocking studies have been carried out in natural environments and aquaculture potential has been investigated. As functional feed additives prebiotics have been reported to have many beneficial effects on growth, survival, immune system, increased absorption in the gut, and improving the general welfare of some fish and crustaceans. The dietary supplementation of the commercial prebiotic Grobiotic®-A (Gb-A) has not been evaluated in *Salmo trutta macrostigma*. This study was planned a preliminary investigation of Gb-A prebiotic supplementation in brown trout diets. In this study, the effects of Gb-A on growth performance, gut structure, and body composition of brown trout were investigated. For this purpose, 600 fish with a mean weight of 0.43 g were used. Gb-A was applied to fish with artemia and feed. After 90 days feeding of the experimental diets, no effect on weight gain, specific growth rate, survival rate, and body structure was observed between the control and Gb-A added groups (1.4% Gb-A and 2.8% Gb-A). However, the intestinal villus length of fish fed 1.4% Grobiotic®-A was higher than fish fed the basal diet and 2.8% Grobiotic®-A. Also, lipid accumulation was observed in both Gb-A supplemented groups in the distal intestine compared to the control group.

Keywords
Grobiotic®-A
Prebiotic
Functional feeds
Artemia
Brown trout

Introduction

Brown trout (*Salmo trutta macrostigma*) is thought to be one of the important salmonid species with high aquaculture potential, economic value, meat quality, attractive appearance and recreational fishery (Kocabas et al., 2011; Güven et al., 2016). Natural distribution fields of Brown trouts are North Africa, Europe, West Asia and Anatolia. It was reported five subspc. in Turkey (Kocabas et al., 2015). On the other hand, it has been reported that brown trout declined in their natural environment due to various factors such as industrial and agricultural pollution and deterioration of habitats and spawning areas. Also, it was disappeared completely in some water sources (Güven et al., 2016; Kocabas et al., 2015). As a result of all this, they have become to critically endangered fish species in inland waters. Consequently, a biological conservation projects have been progressed for *Salmo trutta macrostigma* in Turkey (Bozkurt et al., 2012; TOB, 2020). With this project, both stocking studies are performed to increase the population of brown trout in their natural habitat and researches are carried out on the aquaculture potential (Kocabas, 2011; Demir et al., 2010; Güven et al., 2016). Thus, farming of brown trout reduces pressure on wild population (Güven et al., 2016).

Fish in culture conditions are more vulnerable to diseases in larval stages. Also, they are more susceptible to physical and physiological situations

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(Gonzales Felix et al., 2018). The fact that brown trout has a longer larval period compared to other cultivated trout species brings some difficulties in its cultivation (Demir et al., 2010; Güven et al., 2016).

Proper nutrition is one of the most important factors in the growth and health maintenance of fish. Prepared diets both provide the essential nutrients required for their physiological functions and mediate the intake of other components that may affect the health of the fish (Li and Gatlin, 2004).

Nowadays, interest in environmentally friendly new applications such as immunostimulants, β-glucan, chitin, probiotic, and prebiotics as an alternative to antibacterial drugs to increase growth performance in aquaculture and prevent diseases has increased (Defoirdt et al., 2011; Dimitroglou et al., 2011; Akrami et al., 2012; Ringo et al., 2014, Hoseinifar et al., 2015; Dawood and Koshio, 2016; Doan et al., 2016; Guardiola et al., 2018; Lieke et al., 2020). These substances, also called functional feed additives, have been reported to provide improvement in immunity, feed evaluation and growth performance, although they are added to the diets in very small amounts (Ganguly et al., 2013).

Due to these various advantages, it will be one of the important research areas to identify the factors associated with diseases and optimize health with biotechnological methods in the future (Ringo et al., 2014). One of the prominent methods in this field is adding prebiotics to feeds. The definition of prebiotics, first presented to the scientific world by Gibson and Roberfroid (1995), was updated by FAO and defined as "non-living nutrient components that benefit host health in connection with the regulation of the microbiota" (Hutkins et al., 2016).

Grobiotic®-A (GbA) used in this study is a commercial prebiotic (International Ingredient Corporation, St. Louis, MO, USA) that is a mixture of partially autolyzed brewer's yeast, dried fermentation products, and dairy ingredients (Li and Gatlin, 2004; Burr et al., 2009; Anguiano et al., 2013; Adel et al., 2016; Rossi et al., 2017). Gb-A has been used in diets for several cultured aquatic organisms. Previous researches with many fish species and some animals such as poultry and swine have been shown that Gb-A-A has the potential to provide many benefits including enhanced resistance of disease against some bacterial (Sink et al., 2008; Zheng et al., 2011) and parasitic (Buentello et al., 2010) pathogens, improved growth performance (Li and Gatlin, 2005; Buentello et al., 2010; Adel et al., 2017), enhanced nonspecific immune responses (Buentello et al., 2010; Zhou et al., 2010), and changes in the intestinal morphology (Rossi et al., 2017; Yazıcı et al., 2020).

Several studies have been conducted on brown trout in areas such as reproductive biology, taxonomy, morphological characteristics, determination of stocks, production, and feeding under controlled conditions (Demir et al., 2010; Kocabas et al., 2011; Oz and Dikel, 2015). There are no studies on the effect of the Grobiotic®-A (Gb-A) prebiotic on brown trout. In this study, it was aimed to determine the effects of Gb-A prebiotic on growth performance, body composition and intestinal histological structure of brown trout in the larval stage.

Material and Methods

Experimental Fish and Culture Conditions

The study was carried out at Çamlıyayla Bahce Natural Trout Production Station located in Tarsus district of Mersin province, affiliated to the Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks. In the study, a total of 600 brown trout larvae with yolk-sacs (mean weight: 0.43±0.01 g) were used. The measurement of the tanks was 50x25x15 cm and 50 fish per tank were placed 12 tanks randomly. Springwater was used in the current study. Water parameters (temperature, dissolved oxygen) were regularly measured with YSI 550A dissolved oxygen instrument. Water temperature and dissolved oxygen were measured as 10.3±0.4°C and 7.9±0.2 mg L^{-1}, respectively. This study was carried out in 4 replications and natural photoperiod was applied. Fish were acclimated to the rearing system for 2 weeks before starting the experiment.

Preparation of the Experimental Diets

The Gb-A used in this study was added to diets in three different levels (control 0%, 1.4%, and 2.8%), and four replicates.

Artemia hatching and enrichment process with Gb-A

Incubation of artemia cysts (1 g L^{-1}) (Artemia SepArt EG >250000 np g−1, INVE Aquaculture Inc.) were performed in 150 L tank (roughly 35-36 ppt) filtered by an ultraviolet (UV) filter at 28-30°C with continuous aeration and illumination (Naz, 2008). Following 24 h, the nauplii were collected and washed with tap water. Enrichment process was done in 5 L glass jar. During enrichment, artemia was added at 400 nauplii mL^{-1} with gentle aeration to ensure a homogeneous distribution. The nauplii were enriched with different amounts of Gb-A (1.4 g L^{-1} and 2.8 g L^{-1}). Enrichment was carried out twice, at the beginning of the incubation period (time 0) and after 12 hours (both times half of the total amounts). Each enrichment process was conducted in triplicate (Naz, 2008). No Gb-A was added to the control artemia group.

Preparation of feed diet with Gb-A

Grobiotic®-A (Gb-A) have been added to commercial 500-900 µm sizes granular trout feed (Skretting Turkey,) according to the literature and the manufacturer's recommendations. No Gb-A was added to the control feed group. The addition of Gb-A to feeds was carried out in the fish feeding unit of Iskenderun Technical University, Faculty of Marine Sciences and Technology. The commercial feeds were placed into the 3D-Mixer Alphie1 (Hexagon Product Development Pvt. Ltd. India) with Gb-A and stirred for 30 min at 80 rpm. Feed sizes were adjusted according to fish measurements in 15-day periods. Fish were fed 4 times a day ad libitum. All diets were prepared at the same time and kept in sterile plastic bags at 4°C until used.

Design of the Experiment

Two interconnected feeding trials were carried out as shown in Table 1 using artemia and commercial feed to evaluate different levels of Gb-A in the brown trout diet. Fish larvae were fed with artemia during the adaptation. The larvae that consumed 75% of the yolk-sac was fed with commercial artemia and Gb-A-enriched artemia until the 15.th treatment day. Upwords the 15th day, fish were fed with a decreasing amount of Gb-A enriched artemia and an increasing amount of Gb-A added feed diets every 15 days. After 2 months, only Gb-A added feed diets were given to fish until the end of the experiment (Table 1).

Evaluation of Growth Parameters

At the end of the growth trial, all fish were fasted for 24 h, and then they were weighed and counted for calculation of WG, Spesific Growth rate (SGR), Feed Conversion Ratio (FCR) and survival (Wang et al., 2016). Fish were bulk weighed at the beginning and end of the trial, and survival was followed daily (Gonzales-Felix et al., 2018). Fish were anesthetized with clove oil (5 mg/L). The growth performance parameters of the fish were evaluated on day 0th, 15th, 30th, 45th, 60th,75th, and 90th. The following formulas were used to calculate the growth parameters and feed consumption of fish (Hoseinifar et al., 2013; Gonzales-Felix et al., 2018):

[Weight gain (WG, g) = (final weight – initial weight)]

[Feed conversion ratio (FCR) = weight gain/feed intake]

[Specific growth rate (SGR, % day^{-1}) = (In final weight – In initial weight)/times (days) × 100]

[Survival (%) = (final animal × 100)/initial animal]

Proximate Compositions

Artemia proximate analysis

Artemia samples for the proximate composition analyses were taken, sieved washed with distilled water and immediately stored at -20°C until the analysis stage.

Whole body and diet proximate composition

At the end of the feeding trial, fish in each treatment was group weighed and sampled following 24 h of feed deprivation. Ten representative fish from each tank were euthanized with an overdose of clove oil, frozen at –20°C, and afterwards homogenized for proximate analysis to determine crude protein, lipid, and ash in whole-body tissue (AOAC, 2005).

Table 1. The experimental feeding schedule throughout the rearing period

Treatment Days	Post hatching days	Artemia enriched Gb-A			Diet containing Gb-A		
		0 GB-A (%)	1.4 GB-A	2.8 GB-A	0 GB-A	1.4 GB-A	2.8 GB-A
1th day	40th day	100%	100%	100%	-	-	-
15th day	55th day	75%	75%	75%	25%	25%	25%
30th day	70th day	50%	50%	50%	50%	50%	50%
45th day	85th day	25%	25%	25%	75%	75%	75%
60th day	100th day	-	-	-	100%	100%	100%
75th day	115th day	-	-	-	100%	100%	100%
90th day	130th day	-	-	-	100%	100%	100%

Diet proximate analysis was performed similarly to fish proximate (Rossi et al., 2015).

Histological Analysis

Following the 90 days feeding trial Gastro Intestinal Tract (GIT) samples were obtained to evaluate possible changes in the intestinal gut in response to the dietary treatments. For this, three randomly selected fish per tank was used as previously described (Betiku et al., 2018; Gonzales-Felix et al., 2018). Briefly, fish were euthanized and the digestive system was dissected to obtain the distal section of the GIT. Tissue samples taken from fish were fixed in 10% phosphate buffered formaldehyde. After fixation, manually processed tissue samples were embedded in paraffin and sliced in 4-5 µm using Leica microtome, stained with hematoxylin-eosin (HE) staining method. The slides were examined under a light microscope (Nikon E 600) equipped with a digital camera.

Statistical Analysis

The SPSS software (version 17.0) program was used to evaluate statistical data. Growth parameter, proximate analysis and intestinal histology results were analyzed by one-way analysis of variance (ANOVA). All means are presented with ± standard deviation (SD). Post hoc Duncan multiple range test was used to determine mean differences among the treatment groups. Differences were considered significant at the 95% confidence level ($P < 0.05$).

Results

Growth Performance

As a result of the experiment, it was observed that different growth parameters such as feed conversion rate, specific growth rate, weight gain, and survival rates were found statistically similar and there were no significant differences among the treatments groups ($p > 0.05$) (Table 2). The survival rate in Gb-A supplemented groups were found higher than the control group. but was not significant.

Table 2. Mean and standard deviation (±SD) of initial weight (IW), final weight (FW), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival of different levels of Gb-A on growth performance of brown trout *(Salmo trutta macrostigma).*

Treatments	Initial Weight	Final Weight	Weight gain	SGR	Survival rate	FCR
Control	0.43±0.0	0.96±0.02	0.54±0.02	0.98±0.04	75.20	1.03±0.07
1.4% GB-A	0.44±0.01	0.95±0.08	0.52±0.08	0.93±0.10	78.50	1.02±0.12
2.8% GB-A	0.43±0.01	0.92±0.03	0.48±0.06	0.88±0.08	80.50	1.03±0.06

Proximate analysis

To evaluate the nutritional effectiveness of administered prebiotic on brown trout fry, the biochemical composition of artemia, diet and body composition were analyzed. The results are presented in Table 3, Table 4 and Table 5, respectively.

Artemia proximate analysis

The crude protein, lipid, and ash composition of artemia enriched with Gb-A were analysed (Table 3). The content of protein in artemia increased related to the increase of the Gb-A level. However, while no statistical difference was observed in lipid ratios, a significant decrease was observed in ash ratios in parallel with increased Gb-A addition.

Table 3. Mean and standard deviation (±SD) of protein, lipid, and ash of Gb-A enriched artemia (Values in a column with different letters denote significant difference ($P < 0.05$).

Artemia proximate analysis			
Treatments	Protein	Lipid	Ash
Control	$44.68±1.88^{a}$	$7.74±1.15^{a}$	$7.21±0.31^{a}$
1.4 % Gb-A	$50.91±5.29^{ab}$	$8.24±4.49^{a}$	$5.11±0.35^{b}$
2.8 % Gb-A	$56.84±1.55^{b}$	$9.34±4.69^{a}$	$5.13±0.68^{b}$

Diet proximate analysis

The diet proximate composition showed no statistical differences among treatments (Table 4).

Table 4. Mean and standard deviation (±SD) of protein, lipid, and ash of Gb-A supplemented feed

Diet proximate analysis			
Treatments	Protein	Lipid	Ash
Control	56.33±3.29	16.46±3.53	9.74±0.10
1.4 % Gb-A	56.93±0.34	15.63±0.93	9.76±0.49
2.8 % Gb-A	57.05±0.49	15.29±0.78	9.67±0.10

Body composition

Whole-body proximate composition (protein, lipid, and ash) did not observe any differences among treatments (Table 5). The crude protein content in the whole body of fish tended to increase with increasing Gb-A prebiotic but no significant differences were obtained ($P > 0.05$).

Table 5. Mean and standard deviation (±SD) of protein, lipid, and ash of body composition of brown trout fry (*Salmo trutta macrostigma*) fed with diets containing 0, 1.4, and 2.8% Gb-A for 90 days. (No significant differences were observed among treatment means at P > 0.05)

Fish Proximate analysis			
Treatments	**Protein**	**Lipid**	**Ash**
Control	12.45±1.876	1.26±0.16	2.34±0.33
1.4 % GB-A	13.25±2.25	1.19±0.13	2.17±0.20
2.8 % GB-A	14.15±1.98	1.33±0.12	2.32±0.26

Intestinal Histology

In the distal intestine, fish fed both diets containing 1.4% Gb-A (132.54μm) and 2.8% Gb-A (122.70 μm) had significantly higher villus compared to fish fed with the control diet (98.63 μm) (Figure 1 A, B, C). It was also observed that lipid accumulation was higher in the 1.4% Gb-A and 2.8% Gb-A groups compared to the control group.

Discussion

Numerous studies have been conducted to evaluate the effects of prebiotics including MOS, FOS, seabass (*Dicentrarchus labrax*) (Yazıcı et al., 2020), Li and Gatlin, 2004), Nile tilapia (*Oreochromis niloticus*) (Vechklang et al., 2012; Peredo et al., 2015), cutthroat trout (*Oncorhynchus clarkii lewisi*) (Sealey et al., 2015). On the other hand, it has been suggested in some studies that adding Gb-A to food provides significant improvements in growth performance (Burr et al., 2009; Zheng et al., 2011; Wang et al., 2016; Adel et al., 2017).

Also, the increase in the survival rates of fish has been noticed (Peredo et al., 2015; Adel et al., 2017). Although there were no significant statistical differences among them, the survival rate in the groups fed with feeds containing Gb-A was higher than the control group, in the present study. Besides, significant increases in the survival rate have been achieved following diseases challenge with some bacteria such as *Aeromonas hydrophila* (Zheng et al., 2011), *Flavobacterium columnare* (Sink et al., 2008), *Streptococcus iniae* and *Mycobacterium marinum* (Li and Gatlin, 2005); infectious hematopoietic virus (Sealey et al., 2007), and a parasite *Amyloodinium ocellatum* (Burr et al., 2009).

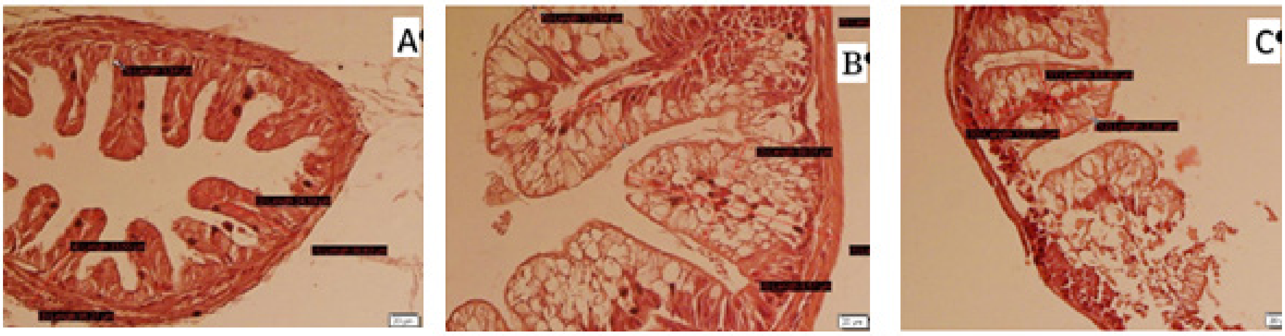

Figure 1. Representation of the distal intestine of *Salmo trutta macrostigma*: A: Control, B: 1.4% GB-A, C: 2.8% GB-A (HE staining, 40 X magnification; bar: 20 μm)

GOS, inulin, Grobiotic-A and its combination with probiotics as dietary supplements in different fish and crustacean species on growth performance, immune function, and disease resistance (Ringo et al., 2010; Torrecillas et al., 2014; Akhter et al., 2015; Sealey et al., 2015; Carbone and Fagio, 2016; Guerreiro et al., 2016). However, the effects of dietary prebiotics on growth parameters and resistance to infectious fish diseases may be inconsistent. Prebiotic administrations are generally performed by addition on diet. In the current study, to our knowledge, this study was the first attempt to research the effects of feeding artemia enriched with Gb-A in brown trout.

In the present study, it was noted that 1.4% or 2.8% dietary supplementation of Gb-A did not affect brown trout's WG, SGR, survival rate and FCR. This was consistent at previous studies on

The body composition of fish plays a vital role as it affects fish growth and survival of cultured species (Hoang, 2019). The whole-body composition is associated with several factors such as life stage, species, and feeding. The nutritional and health status of species in aquaculture can often be predicted by determining their body composition such as protein and lipid. It was indicated that ingredients of diet and their nutritional values affect the effectiveness of supplemented prebiotics of fish in different rearing conditions (Ghafarifarsani et al., 2020). It is important to get information about the proximate composition of fish and the factors affecting the proximate composition may provide determine of fish health, and assessment of efficiency of transfer of nutrients from the feed to the fish. So it may be possible to predictably modify carcass composition (Shearer, 1994).

In the present study, no significant differences were observed in whole-body composition (Table 5) of brown trout after 90 days feeding with 1.4% and 2.8% Gb-A supplemented diets. These results were consistent with previous studies in Nile tilapia (Zheng et al., 2011; Vechklang et al., 2012), and sturgeon (Adel et al., 2017).

Contrary to our findings, Azari et al. (2013) reported that proximate analysis showed significant ($P<0.05$) differences in rainbow trout muscle protein, lipid, ash, and moisture contents of all the treatments. They suggested that the inclusion of Gb-A (%1-3) in rainbow trout diets may help to positively change in body composition by reducing the fat content.

It has been proposed that changes in intestinal morphology have major impacts on nutrient absorption and metabolism, as higher intestinal villi length may increase the absorption of surface area (Bae et al., 2020). It has been indicated that some of the prebiotics in the diets may cause significant differences in gastrointestinal morphology in some fish and crustacea (Anguiano et al., 2013). It is stated that structural changes in the intestine may be related to the production of short-chain fatty acids by the microbial fermentation of prebiotics. In addition, the gut surface is one of the important parameters used to assess the health of aquaculture species (Hoang, 2019).

In the present study, the addition of prebiotics affected the intestinal histological structure. In the microphotographs of the distal section of the intestines of the brown trout fries, it was measured that the villi were both longer and wider in the experimental group than the control group. Moreover, it was observed that lipid accumulation was higher in 1.4% Gb-A and 2.8% Gb-A compared to the control group. The villus length was 98.63 µm in the control group, while it was 132.4 µm and 122.70 µm in the 1.4%Gb-A and 2.8% Gb-A groups, respectively. These results are similar with Anguiano et al. (2013) who reported an increase in intestinal villi of red drum (S. ocellatus) fed for 4 weeks with diets containing 2% Gb-A. However, Yazıcı et al. (2020) reported that the increase of the Gb-A rate in the diet negatively affects the histological structure of the seabass intestine. Contrary to our findings, no noticeable changes in the distal intestine of red drum fed with diets supplemented with different prebiotics (Zhou et al., 2010). It has been suggested that some of the differences in fish may be related to dietary preferences, gut microbiota, and changes in gut structure and length, which may explain the observed differences in fish (Peredo et al., 2015).

Conclusion

In conclusion, results of this study showed that the weight gain, whole-body proximate composition and survival of brown trout after 90 days of feeding were not significantly affected by dietary supplementation of 1.4% and 2.8% Gb-A. However intestinal structure was affected in the treatment groups.

This research has been a preliminary investigation for the Grobiotic®-A prebiotic supplementation in brown trout diets. Further investigation of the effects of Gb-A on diverse life stages, challenges to stress factors or pathogens, intestine microbiota, and immunity-related gene expression is required to fully elucidate the effectiveness of Gb-A within different supplemental levels.

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COMPLIANCE WITH ETHICAL STANDARDS

Authors' Contributions

Authors contributed equally to this paper.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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