Journal of Agricultural Sciences
(Tarim Bilimleri Dergisi)
J Agr Sci-Tarim Bili
e-ISSN: 2148-9297
jas.ankara.edu.tr
JAS

Effects of Dietary Sage, Myrtle and/or Probiotic Mixture on Growth, Intestinal Health, Antioxidant Capacity, and Diseases Resistance of *Oncorhynchus mykiss*

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ARTICLE INFO
Research Article
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Received: 24 May 2022 / Revised: 21 Dec 2022 / Accepted: 21 Dec 2022 / Online: 25 Mar 2023

Cite this article
ÖZİL Ö, DİLER Ö, KAYHAN H K, KÖK TAŞ T, SEYDİM Z B, DİDİNEN B I (2023). Effects of Dietary Sage, Myrtle and/or Probiotic Mixture on Growth, Intestinal Health, Antioxidant Capacity, and Diseases Resistance of *Oncorhynchus mykiss*. *Journal of Agricultural Sciences (Tarim Bilimleri Dergisi),* 29(2):721-733.
DOI: 10.15832/ankutbd.1120481

ABSTRACT
It is widely known that the use of medicinal plants and probiotics as feed additives has a positive effect on growth, non-specific immune system, and resistance to diseases in aquaculture. This study examines the effects of dietary supplementation with sage (*Salvia officinalis*) and myrtle (*Myrtus communis*), alone or in combination with a probiotic mixture (PM) on growth, intestine microflora and histology, some antioxidant enzymes activities in the muscle tissues of rainbow trout (*Oncorhynchus mykiss*) and disease resistance against *Vibrio anguillarum*. For this purpose, fish were fed with a control diet of 1% sage, 1% myrtle, 1.1% probiotic mixture, 1% sage +1.1% PM and 1% myrtle+1.1% PM supplemented diets for 60 days. At the end of the trial, the fish fed the diets supplemented with myrtle and sage + PM showed a positive effect on feed conversion ratio. According to the histological assessment, the villi length, villi width and goblet cell numbers in the intestines of fish in all groups increased compared to the control. Superoxide dismutase activity in the muscles of fish in the PM group was higher than the fish in the other groups ($p<0.05$). The malondialdehyde activity was unaffected with the exception of the fish in the sage group ($p<0.05$). The lactic acid bacteria count in the intestines increased in fish fed the sage + PM ($p<0.05$). Fish fed the diets supplemented with sage + probiotic mixture, probiotic mixture, myrtle + probiotic mixture, and myrtle saw a significant reduction in mortality (0-32.5%) due to V. *anguillarum* compared to the control (63.2%) ($p<0.05$). In conclusion, the use of probiotics, sage and myrtle in combination as a feed supplement showed a positive effect on the growth performance, intestinal microflora and histology, and antioxidant enzymes activities and disease resistance in rainbow trout.

Keywords: *Salvia officinalis*, *Myrtus communis*, Probiotic, Histology, *Vibrio anguillarum*, Rainbow trout

1. Introduction

The aquaculture sector has seen a rapid expansion across the world in recent decades (Guardiola et al. 2017). In Turkey, almost all of freshwater farming is based on rainbow trout production (Okumuş 2002). The benefits of better growth rate, better feed utilization rate and enhancement against diseases on fish culture will provide high added value to the country. In addition, intensive culture has led to the stress and the outbreak of diseases (Kennedy et al. 2016) in fish. Antibiotics and chemotherapeutics agents are extensively used in the treatment of diseases in aquaculture. However, these practices have not only negatively affected the environment (Santos & Ramos 2018; Srichaiyo et al. 2020), but also formed antimicrobial resistance in bacteria. For this reason, the use of medicinal plants to manage fish pathogens is an alternative and current practice. Medicinal plants are being used in aquaculture not only as chemotherapeutics but also as feed additives, as they contain a wide variety of chemical compounds (Awad & Awaad 2017). Probiotic products may provide broad-spectrum and greater non-specific disease protection (Lara-Flores 2011). There is evidence to suggest that probiotics in aquaculture may prove effective in improving growth performance, immunostimulation and increase disease-resistance (Balcazar et al. 2006).

Myrtle (*Myrtus communis* L.) is a Mediterranean evergreen shrub that has been used since ancient times for medicinal, food and spice purposes. The dried leaves of this herb have volatile oils which contain 1.8-cineole, linalool, linalyl acetate, terpinolene, tannins and flavonoid compounds. The leaves and fruits have long been used in Turkish folk medicine for antiseptic purposes when healing wounds, and in the treatment of prostatitis, bronchitis, sinusitis, and colds (Keven-Karademir & Avunduk 2015). Previous studies have reported the growth promotion (Taee et al. 2017a), antimicrobial (Taee et al. 2017b), anaesthetic (Al-Niaeem et al. 2019), antioxidant (Safari et al. 2017) properties of myrtle in fish.

Sage (*Salvia officinalis* L.) is a plant in the Lamiaceae family (Carović-StanKo et al. 2016), it contains some components (α, β-pinene, 1,8-cineole, borneol, and α, β-thujone) which have antibacterial (Hać-Szymańczuk et al. 2014, 2015) and antioxidant (Wojdyło et al. 2007; Roby et al. 2013) properties (Bernotienė et al. 2007). A number of studies have extensively analysed the effects of sage oil on growth in different fish species (Salomón et al. 2020), immune response (Terzioğlu & Diler 2016), meat quality (Mehdizadeh et al. 2019) and blood parameters (Aydin & Harmantepe 2018).

Over the past decade, particularly in aquaculture, research has focused on the application of using aromatic plants to replace subtherapeutic antibiotics in growth promoters, disease control, immune response, and disease resistance of various fish species. In addition, studies have shown that the use of medicinal plants in a synbiotic with probiotics increases these effects even more (Abdallah et al. 2022). However, studies in aquaculture on this subject are very limited.

This study investigates the effects of dietary supplementation with sage and myrtle plants alone as well as in combination with an indigenous and exogenous probiotic mixture (PM) composed of four lactic acid bacteria, Bifidobacterium spp. and one yeast strain on rainbow trout growth, intestinal microflora and histology, antioxidant enzyme activities and disease resistance against *Vibrio anguillarum*.

2. Material and methods

2.1. Preparation of experimental diets

The PM was prepared to contain lactic acid bacteria (*Lactobacillus* spp., *Lactococcus* spp., *Lactobacillus acidophilus*), *Bifidobacterium* spp., one yeast strain (*Kluyveromyces marxianus*) was isolated from kefir (Kök Taş et al. 2012; Gümüş et al. 2017) and *Lactobacillus sakei* from rainbow trout intestines (Didinen et al. 2018). The lactic acid bacteria were grown on De Man, Rogosa and Sharpe (MRS) Broth (Merck 110661) and incubated for 24 hr at 25 °C. The cells were collected by centrifugation at 5,000 g for 15 min at 4 °C. Experimental diets were formulated based on the study of New 1987 (Table 1). Different probiotic bacteria species were added to feed at a rate of 1.1% with sunflower oil (0.05 mL kg^{-1}) to provide 1×10^8 CFU g^{-1}.

Myrtle berries and sage leaves samples were obtained from a commercial company that has official production permission in Isparta and dried at room temperature and then powdered. The powder was mixed with sunflower oil (0.05 mL kg^{-1}) and added at a rate of 1% to the feed.

Table 1- Formulation of the experimental diets (g kg^{-1})

Feed ingredients	***Groups***					
	Control	***Myrtle (1%)***	***Myrtle (1%) + PM***	***Sage (1%)***	***Sage (1%) + PM***	***PM***
Fish meal[1]	350	350	350	350	350	350
Soybean meal[2]	300	300	300	300	300	300
Wheat gluten[3]	50	50	50	50	50	50
Wheat meal[4]	124.95	114.95	103.95	114.95	103.95	113.95
Fish oil[5]	80	80	80	80	80	80
C vitamin[6]	5	5	5	5	5	5
Vitamin premix[7]	20	20	20	20	20	20
Mineral premix[8]	10	10	10	10	10	10
Pellet binders[9]	40	40	40	40	40	40
Antioxidant[10]	5	5	5	5	5	5

Table 1- continued

Feed ingredients	***Groups***					
	Control	***Myrtle (1%)***	***Myrtle (1%) + PM***	***Sage (1%)***	***Sage (1%) + PM***	***PM***
Others[11]	15	15	15	15	15	15
Additions to standard diet						
Plant powder	-	10	10	10	10	-
Lactobacillus spp.	-	-	2	-	2	2
Lactococcus spp.	-	-	2	-	2	2
Lactobacillus acidophilus	-	-	2	-	2	2
Bifidobacterium spp.	-	-	2	-	2	2
Yeast strain	-	-	2	-	2	2
Lactobacillus sakei	-	-	1	-	1	1
Sunflower oil	0.05	0.05	0.05	0.05	0.05	0.05

[1-6]Abalioglu Feed Factory, Torbalı-Izmir/Turkey
[7]Vitamin premix.; per kg, 4,000,000 IU vitamin A, 480,000 IU vitamin D3, 40,000 mg vitamin E, 2,400 mg vitamin K3, 4,000 mg vitamin B1, 6,000 mg vitamin B2, 40,000 mg niacin, 10,000 mg calcium D-pantothenate, 4,000 mg vitamin B6, 10 mg vitamin B12, 100 mg D-biotin, 1,200 mg folic acid, 40,000 mg vitamin C and 60,000 mg inositol.
[8]Mineral premix.; per kg 23,750 mg Mn, 75,000 mg Zn, 5,000 mg Zn, 2,000 mg Co, 2,750 mg I, 100 mg Se, 200,000 mg Mg.
[9]Pellet binders; lignosulfonate.
[10]Antioxidant; ethoxyquin.
[11]Others; choline chloride, methionine + cystine.
PM: Probiotic mixture

The experimental groups were be formed as sage (group I), myrtle (group II), PM (group III), sage + PM (group IV) and myrtle + PM (group V) and control (group VI).

2.2. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of plant powder

A GC-MS analysis was performed to determine the phenolic content of the plants and the GC-MS analyses of the plants were carried out using a Hewlett-Packard 6890 series gas chromatograph fitted with a flame ionization detector, CPWax 52CB capillary column (50 m×0.32 mm; film thickness ¼ 0.25 Im). Pure helium gas was used as the carrier gas at a constant flow rate of 40 mL min^{-1}. The injection quantity was 1 µL, and the injector and detector temperature was maintained at 240 °C. The column oven temperature was set at 60 °C and was raised by 2 °C per min up to 220 °C. The final temperature was maintained at 220 °C for 20 min. Relative percentage amounts were calculated from chromatograms from the Turbo Crom Navigator computer program.

2.3. Fish and experimental conditions

The experiments were performed in systems with a water flow rate of 12 L min^{-1}. The water quality parameters were measured as temperature 12±2 °C, dissolved oxygen 7.4 mg L^{-1} and pH 7.3. A total of 600 (6 groups with two replicates, 50 fish per tank) average 45.44±3.11-50.65±3.26 g rainbow trout (*Oncorhynchus mykiss*, Walbaum) were randomly distributed into 600 L tanks. The experimental fish were fed daily at 2% of their biomass for 60 days. Throughout the study, the fish were weighed at 2 week intervals and their weight was determined and the amount of feed to be given was regularly updated according to their body weight. The welfare of the fish was performed according to the ethical standards of the national guidelines approved by the Isparta Applied Sciences University Animal Care and Use Committee (decision number: 001, date: 24.09.2020).

2.4. Detection of the growth performance

Growth performance was evaluated based on the following formulas (De Silva & Anderson 1995):

Weight gain (WG, g) = W2-W1;

Specific growth rate (SGR, %day) = 100 x (Ln W2-Ln W1)/T;

Feed conversion ratio (FCR) = (feed intake, g)/(weight gain, g);

Condition factor (CF) = 100 x (body weight, g)/(standard length^3, cm);

Survival rate (SR, %) = 100 x (Nf / Ni)

Feed intake (FI, g day^{-1}) = (total consumed of feed, g)/(number of fish)

W1 (Initial weight), W2 (Final weight), T (Number of days in the feeding period), Ni (Initial number of fish) and Nf (Final number of fish), respectively.

2.5. Enumeration of intestinal microbiota

To determine the effect of dietary plant powder and probiotics on the bacterial population of the intestine, 6 fish samples were taken from each experimental group at the end of 60 days. After the fish was anesthetized (clove oil, 50 mg L^{-1}), the skin was wiped with alcohol and digestive tract samples were taken by ventral incision. Samples were created by homogenizing whole intestinal samples in phosphate buffered saline (PBS) and tenfold serial dilutions of these samples were prepared and spread on Man, Rogosa and Sharpe Agar to obtain lactic acid bacteria. The plates were incubated at 37 °C for 48 hours, after which the plate count agar were incubated at 30 °C for 48 hours to acquire a total count of the aerobic bacteria. Following this, the bacteria colonies were counted and averaged for each sample (Giannenas et al. 2012).

2.6. Histological examination of intestines

At the end of the study, after anaesthesia (clove oil, 50 mg L^{-1}), a necropsy was performed on 6 fish from each experimental group. Following the aseptic dissection, the intestine in its entirety was removed. The anterior intestinal tissue was then excised and samples taken and fixed in 10% neutral formalin solution and processed by automatic tissue processing equipment (Leica ASP300S). The intestine samples were embedded in paraffin, and 5 µm longitudinal sections of the intestine were cut using a rotary microtome. Then, intestine sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

In order to evaluate the morphometric changes of the intestines, the length and width of each villi were measured at 40x under a microscope. The morphometric evaluation was carried out using the Database Manual Cell Sens Life Science Imaging Software System. The mucous cells (goblet cells) in the anterior part of the intestine were counted for each fish and the mucous cell counts were reported as mean number ± standard deviation per 100,000 µm^2 of epithelial section area (Heidarieh et al. 2013).

2.7. Antioxidant activity in tissues

A total of 20 fish from each experimental group were decapitated under anaesthesia (clove oil, 50 mg L^{-1}) at day 60 after the feeding trial. Muscle samples were taken and washed with physiological saline. A 3 mL Tris-HCl buffer containing 0.25 mol L^{-1} sucrose was adjusted to pH 7.3 and mixed with the tissue sample and stored at -80 °C. The muscle tissues were homogenized in a motor-driven tissue homogenizer with phosphate buffer (pH 7.4). Unbroken cells, cell debris, and nuclei were sedimented by centrifugation at 2,000 g (rpm) for 10 min. The levels of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and catalase (CAT) were defined in the supernatants. The determination of SOD activity was predicted in the supernatant according to the method described by Aebi (1974) and is referred to in kilounits per gram protein. The MDA levels in the tissues were detected from the homogenate by following the double heating method (Draper & Hadley 1990); the concentration of MDA is obtained as micromoles per gram protein in the muscle tissue. SOD, CAT activities and MDA levels were determined using a spectrophotometer (Shimadzu UV-1601).

2.8. Bacterial challenge

The pathogenicity of the *V. anguillarum* strain in rainbow trout was confirmed by intraperitoneal injecting (i.p.) and reisolating pathogens from diseased fish prior to the experiments. After 60 days of feeding, the experimental infection was performed by i.p. injection under anaesthesia (clove oil, 50 mg L^{-1}). The *V. anguillarum* pathogen was inoculated on Tryptic Soy Broth medium and incubated at 25°C for 24 hours. The cells were harvested by centrifugation (2,000f), washed with PBS and re-suspended in PBS. 40 fish from each experimental group were intraperitoneally injected with *V. anguillarum* with 0.1 ml volumes of bacterial suspension adjusted to 2.0x105^5 CFU mL^{-1} (LD_{50} dose) (Diler et al. 2017). Mortalities were recorded daily and any moribund fish were examined bacteriologically. Afterwards, the relative percent survivals (RPS) were calculated according to Amend (1981).

2.9. Statistical analysis

For the evaluation of differences in growth parameters including final weight, SGR, FCR and feed intake, the analysis of covariance (ANCOVA) was used with the incorporation of initial average weights as a covariate. The influence of treatments on survival rates, lactic acid bacteria and total bacteria counts in the intestines, antioxidant activity, mortalities, RPS values, length and width of the villi, goblet cells counts of intestines between the experimental groups were analyzed by one-way analysis of variance (ANOVA). SPSS 18.0 software was used to analyze the data. The significant variables were discriminated among the treatments using the Duncan test. In the statistical analysis, $p<0.05$ was considered as the significance level for growth parameters, survival rates, lactic acid bacteria and total bacteria counts in the intestines, antioxidant activity and mortalities and RPS values while $p<0.001$ was for the villi length, width and goblet cell numbers of the intestines.

3. Results

3.1. Chemical composition of medicinal plants

In this study, the major compounds in myrtle; hexanol 32.84%, 1.8-cineole(=eucalyptol) 31.62%, camphor 8.73%, alpha pinene 8.39% and limonene 4.63%; in sage; 1.8 cineole (56.98%) and camphor (21.15%) were determined (Table 2).

Table 2- Components of sage and myrtle

Compound	***Myrtle (%)***	***Sage (%)***
Alpha Pinene	8.39	1.93
Hexanal	32.84	-
Limonene	4.63	0.46
1.8-Cineole	31.62	56.98
1-Pentanol	2.05	-
p-Cymene	2.41	0.91
2-Heptenal	1.85	-
1-Hexanol	2.82	-
7-octen-4-ol	2.82	0.39
Camphor	8.73	21.15
Alpha-Terpineol	1.84	1.31
Camphene	-	1.74
Hexanal	-	0.43
Beta-Pinene	-	0.39
Beta-Myrcene	-	1.14
2-Hexenal	-	0.41
3-Hexen-I-ol, (Z)		0.20
Alpha-Thujone	-	1.10
Beta-thujone		0.69
Bomyl acetat	-	3.43
4-Terpineol	-	0.29
Trans-Caryophyllene	-	3.48
Aromadendrene	-	0.26
Linalyl oxide	-	0.42
Alpha-Humulene	-	0.48
Terpinyl acetate	-	0.27
Borneol	-	2.13

3.2. Growth parameters

In this study, the difference was not statistically significant between the groups in terms of final weight, weight gain, condition factor, specific growth rate, feed intake, and survival rate ($p>0.05$). The FCR values of the fish fed the diet with myrtle and sage + PM were significantly lower than the fish in the other groups and the control ($p<0.05$) (Table 3).

Table 3- Growth performance of the rainbow trout fed containing plants without or with probiotic bacteria

	Experimental groups					
	Control	***Myrtle (1%)***	***Myrtle (1%) + PM***	***Sage (1%)***	***Sage (1%) + PM***	***PM***
W1 (g)	46.26±0.91	45.44±3.11	50.50±2.97	46.62±0.41	50.65±3.26	46.80±0.16
W2 (g)*	72.85±3.79	76.69±5.65	79.95±4.90	75,24±6.33	76.12±0.05	75.20±7.46
WG (g)*	26.58±4.70	31.25±2.54	29.46±1.92	28.62±5.92	25.47±3.31	28.41±7.30
CF	1.23±0.15	1.29±0.44	1.24±0.18	1.22±0.11	1.27±0.14	1.25±0.09
FCR*	1.12 ± 0.17^{ab}	0.78 ± 0.01^{c}	1.29 ± 0.01^{a}	1.21 ± 0.02^{ab}	0.92 ± 0.13^{bc}	1.25 ± 0.07^{ab}
SGR (% day)*	0.76±0.12	0.87±0.01	0.77±0.00	0.79±0.13	0.68±0.11	0.79±0.16
FI (g day^{-1})*	0.49±0.01	0.40±0.04	0.63±0.01	0.57±0.10	0.39±0.09	0.58±0.01
SR (%)	100.00±0.00	97.50± 0.70	92.00±5.65	100.00±0.00	96.00±5.65	100.00±0.00

*Actual average values are shown but they were analyzed with ANCOVA using initial mean weights as covariate.
Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$).
PM: Probiotic mixture, W1: Initial weight, W2: Final weight, WG: Weight gain, CF: Condition factor, FCR: Feed conversion ratio, SGR: Specific growth rate, FI: Feed intake, SR: Survival rate

3.3. Microbiological analysis

The lactic acid bacteria count in the intestine of fish in the sage + PM group was found to be higher than the fish in other groups. The total bacteria count in the intestine of fish fed with PM, sage and myrtle + PM groups were higher than the fish in other groups (Table 4).

Table 4- Lactic acid bacteria and total bacteria counts of fish in groups

Groups	***Lactic acid bacteria count (log CFU g^{-1})***	***Total bacteria count (log CFU g^{-1})***
Myrtle (1%)	2.50 ± 0.06^{b}	1.27 ± 0.43^{b}
Myrtle (1%) + PM	2.40 ± 0.37^{b}	6.05 ± 1.79^{a}
Sage (1%)	2.51 ± 0.02^{b}	7.26 ± 3.06^{a}
Sage (1%) + PM	3.13 ± 0.00^{a}	2.60 ± 1.58^{b}
PM	2.27 ± 0.24^{b}	6.25 ± 0.21^{a}
Control	2.14 ± 0.00^{b}	3.04 ± 1.14^{b}

Data are presented as the means ± standard devation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture

3.4. Histology of intestine

Based on a microscopical examination of intestine samples in fish sampled from all groups, there were no pathological findings (Figure 1). In general, an increased villi length, width and numbers of goblet cell were observed in fish from all experimental groups compared to the control group ($p<0.001$) (Table 5). The best group on intestinal villi length and villi width of the fish was the myrtle + PM, while the best group in goblet cell numbers was determined to be the sage + PM group. Probiotics had an additional effect in improving the effects of the myrtle and sage groups (Figure 1).

Table 5- Villi length, villi width and goblet cell numbers of fish intestines in groups

Groups	*Villi length (µm)*	*Villi width (µm)*	*Goblet cell count*
Myrtle (1%)	588.75±15.13^{b}	92.75±2.50^{b}	101.50±1.29^{b}
Myrtle (1%) + PM	611.50±7.04^{a}	105.75±4.34^{a}	99.75±1.70^{b}
Sage (1%)	578.50±8.50^{b}	91.25±1.70^{b}	98.00±2.16^{b}
Sage (1%) + PM	595.25±4.11^{ab}	101.25±3.86^{a}	109.00±1.82^{a}
PM	591.50±8.69^{ab}	102.25±2.06^{a}	102.00±5.47^{b}
Control	481.75±35.10^{c}	85.25±5.18^{c}	92.25±3.59^{c}

Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture

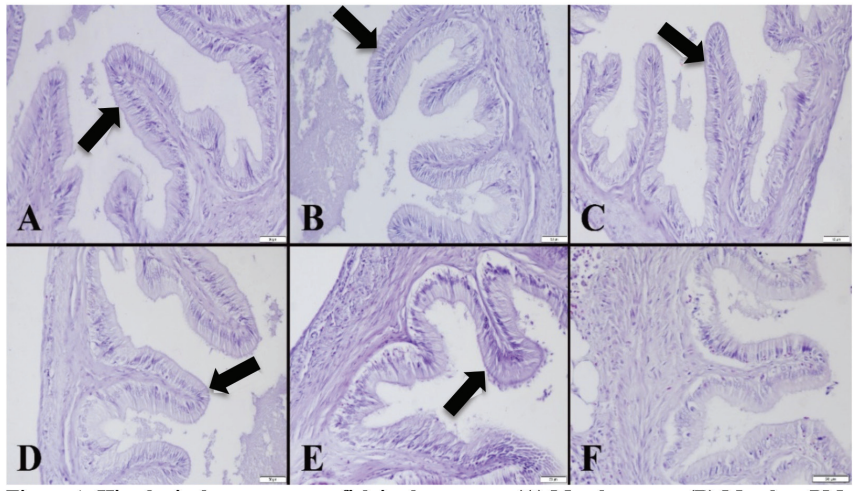

Figure 1- Histological gut structure fish in the groups; (A) Myrtle group, (B) Myrtle + PM group, (C) Sage group, (D) Sage + PM group, (E) PM group, (F) Control group, H&E, Bars=50 µm. The increase in villi length, villi width and in the number of goblet cells in the intestines of fish is indicated by the arrow

3.5. Antioxidant activity

The MDA level was unaffected with the exception of the fish in the sage group ($p<0.05$). In addition, the SOD activity of fish in the PM group was higher than those in other groups ($p<0.05$). CAT activity was not affected regardless of the treatment (Table 6).

Table 6- Antioxidant activity of fish in groups

Groups	*MDA*	*CAT*	*SOD*
Myrtle (1%)	0.48±0.37^{ab}	0.16±0.07	1.54±0.20^{bc}
Myrtle (1%) + PM	0.26±0.12^{b}	2.49±4.78	1.75±0.33^{b}
Sage (1%)	0.71±0.12^{a}	0.40±0.24	1.30±0.09^{c}
Sage (1%) + PM	0.29±0.10^{b}	0.36±0.42	1.34±0.15^{c}
PM	0.39±0.20^{b}	0.25±0.18	2.06±0.08^{a}
Control	0.29±0.10^{b}	0.36±0.32	1.74±0.14^{b}

Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture, MDA: Malondialdehyde, CAT: Catalase, SOD: Superoxide dismutase

3.6. Diseases resistance against V. anguillarum

The statistical analysis demonstrated that fish fed with diets containing sage + PM, PM, myrtle + PM, myrtle at 21 days had significantly lower levels of mortality than those in the sage and control groups ($p<0.05$) (Figure 2). The RPS values were calculated as 100, 72.36, 60.38 and 48.62 in fish fed with diets containing sage + PM, PM, myrtle + PM, myrtle, respectively (Table 7). When resistance against *V. anguillarum* was evaluated, it was determined that the highest effect with 100% RPS value was in fish fed with diets containing sage + PM.

Table 7- Mortality and RPS values of fish in experiment groups challenged with *V. anguillarum*

	Mortality (%)	***RPS (%)***
Myrtle (1%)	32.50±3.53^{b}	48.62±4.50^{d}
Myrtle (1%) + PM	25.00±3.21^{c}	60.38±5.92^{c}
Sage (1%)	52.77±3.92^{a}	16.54±4.45^{e}
Sage (1%) + PM	0.00±0.00^{e}	100.00±0.00^{a}
PM	17.50±3.53^{d}	72.36±5.01^{b}
Control	63.20±1.33^{a}	-

Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture, RPS: Relative percent survivals

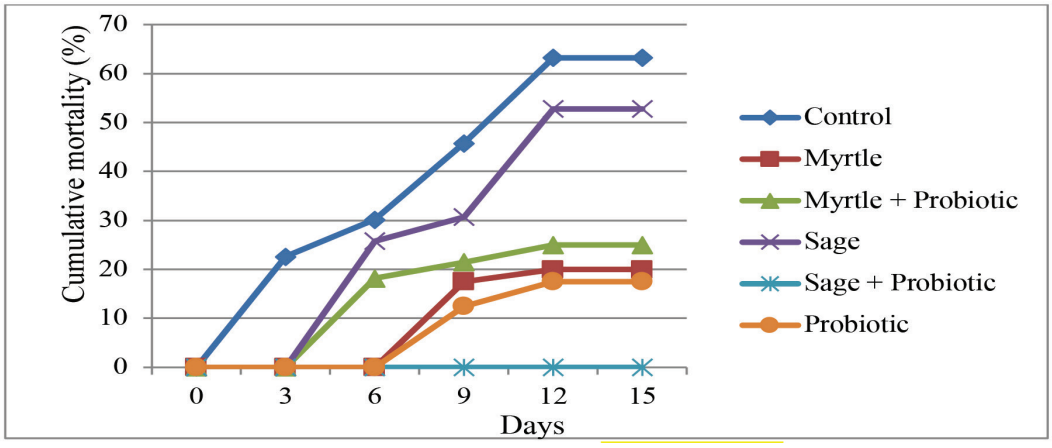

Figure 2- Fish cumulative mortality (%) against *V. anguillarum* among the groups

4. Discussion

This study found that a dietary supplementation with sage and myrtle, alone or in combination with PM increased the final fish weight but the difference was not found to be statistically significant ($p<0.05$). In a 2015 study, Sönmez et al. (2015) noted that a dietary inclusion of sage oil was effective in enhancing the growth of rainbow trout. El-Kholy (2012) also noted an increase in weight gain and feed efficiency in a tilapia hybrid (*Oreochromis niloticus* × *Oreochromis aureus*) fed with *S. officinalis* leaf powder at 150 and 300 mg kg^{-1} feed for 90 days. In contrast, Aydin & Harmantepe (2018) have reported that the administration of sage oil to tilapia (*Oreochromis niloticus*) diets (0, 0.25, 0.5 and 1%) reduced feed intake, specific growth rate, weight gain and final weight. In addition, Dadras et al. (2019) noted that the use of sage extracts (30, 60 and 120 mL kg^{-1} feed) in juvenile sturgeon (*Huso huso)* had no affect growth performance. The different results in growth performance may be related to the levels of herbal plants tested.

Taee et al. (2017a) noted a decrease in FCR in rainbow trout fed with myrtle powder. Mohamadi et al. (2016) also stated that feeding rainbow trout with 300 mg kg^{-1} of myrtle essential oils led to a decrease in FCR. In addition, Safari et al. (2017) reported that dietary myrtle at 20 g kg^{-1} improved the growth-related gene expressions in zebrafish (*Danio rerio*). Similarly, in the present study, it was determined that myrtle and sage + PM was more effective on the FCR values than other groups as well as the control.

The presence of autochthonous probiotic bacteria in the diet has changed the microbial metabolism of fish, which enhances growth performance by stimulating exogenous enzyme production (Bhatnagar et al. 2012). Similarly, Bhatnagar et al. (2012) determined that probiotic bacterium *Bacillus coagulans* was incorporated in different levels of *Mentha piperita*. Their results showed low FCR in the group fed diets with *M. piperita* and *Bacillus coagulans.*

The positive effects of lactic acid bacteria on growth were determined in different fish species such as *Lactobacillus acidophilus* in grass carp (Wang 2011); *Lactobacillus curvatus* in Persian sturgeon (*Acipencer percisus*), *Leuconostoc mesenteroides* in beluga (*Huso huso*) (Askarian et al. 2011); *Lactobacillus plantarum* in tilapia (Yu et al. 2017); *Lactobacillus casei* in *Barbus grypus* (Mohammadian et al. 2017). In present study, the final weight in rainbow trout fed with PM was found to be higher than in the control group; the difference, however, was not statistically significant ($p<0.05$).

Nutraceuticals such as probiotics, prebiotics, synbiotics, medicinal plants and immunostimulants affect the gut microbiota. Probiotics which stick with the mucosal epithelium of intestine and prevents pathogens colonization are a vitally importance modulation of intestinal microbiota (Hoseinifar et al. 2018). Giannenas et al. (2012) reported that the total counts of aerobic gut bacteria were not affected whereas the levels of *Lactobacillus* spp. were decreased by supplementation of thymol to the diet for 8 weeks. In the present study, the lactic acid bacteria counts in the intestine were unaffected by sage and myrtle groups in rainbow trout. However, the lactic acid bacteria counts of fish in the sage + PM group were found to be higher than those in other groups. Merrifield et al. (2010), concluded that the addition of different types of probiotics (*B. licheniformis*, *B. subtilis* and *E. faecium*) to the feed significantly increased the intestinal microbiota. In this study, the results were compatible with the literature, and it was determined that the fish in the group supplemented with PM increased the total number of bacteria in the intestinal microbiota. These findings suggest that a dietary inclusion of phytogenic products combined with PM may have a positive effect on the intestinal populations of rainbow trout.

The combination of probiotics and herbal products as an alternative disease control strategy is provided by improvement of hematological and biochemical parameters, disease resistance to pathogens (Ringø & Song 2016). Probiotics have been used to fight pathogens by producing inhibitory compounds such as bacteriocins, lysozymes, organic acids, proteases, hydrogen peroxide, diacetyl and other inhibitory chemicals (Karmakar et al. 2012). Van Doan et al. (2016) indicated that a dietary combination of *Lactobacillus plantarum* with Jerusalem artichoke (*Helianthus tuberosus*) in pangasius catfish (*Pangasius bocourti*) improved the serum lysozyme and respiratory burst activities, phagocytic index and disease resistance against *A. hydrophila* when compared to control fed fish. Harikrishnan et al. (2011) reported that the supplementation of *Lactobacillus sakei* and *Scutellaria baicalensis* improved the haematological, innate immune response and resistance against *Edwardsiella tarda* in barred knifejaw (*Oplegnathus fasciatus)*. Similarly, in the present study, the use of PM alone and in combination with sage in rainbow trout feeding improved disease resistance to *V. anguillarum.* Medicinal plants may have provided a protection against this pathogen due to the phenolic compounds that are known to restrict or inhibit the growth of bacteria (Dorman & Deans 2000). The antimicrobial activity of sage and myrtle was based on the 1,8-cineole component (Metin et al. 2020).

While nutritional components affect intestinal morphology, natural feed additives improve growth performance, feed efficiency and intestinal histology (Giannenas et al. 2012). Therefore, it can be supposed that the enhancement of goblet cell density could result in a higher defence mechanism against pathogens in rainbow trout. Diler & Görmez (2019) observed that the chemical components of *Artemisia absinthium* L. and *Artemisia campestris* L. are strongly associated with the efficiency of intestinal morphology in rainbow trout and the administration of *A. absinthium* L. to the diet of trout presented led to increase in higher goblet cells, villus length and width. Studies have shown that increasing the length and width of the villi increases the absorption in the intestines (Dimitroglou et al. 2010), and that the presence of goblet cells is an important indicator of intestinal health (Elsabagh et al. 2018). Similarly, the present study found increased villi length, villi width and goblet cell numbers of fish in all experimental groups compared to fish in the control group.

SOD and CAT are enzymes that play an important role in antioxidant defence mechanisms in biological systems (Livingstone 2001; Ritola et al. 2002; He et al. 2015). Safari et al. (2017) reported that dietary myrtle at 20 g kg^{-1} improved antioxidant (*SOD* and *CAT*) enzymes gene expression in zebrafish. In contrast, in the present study, the use of myrtle in rainbow trout feed did not affect SOD and CAT activities in muscles. This difference may be due to the different fish species and the doses of myrtle used in the studies. MDA, which is one of the end products of lipid peroxidation caused by free oxygen radicals, is commonly known as a marker of oxidative stress and antioxidant status. The increase in MDA levels is a crucial indicator of cell membrane injury (Yagi 1984). Our study was determined that the MDA activity in muscles of fish in the myrte+PM group was lower than fish in the other groups. The results are in line with the MDA activity of aqueous methanolic extract of pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*)

(Sönmez et al. 2022), sage and thyme oil in rainbow trout (Sönmez et al. 2015b) and mucuna (*Mucuna pruriens*) seed extract in *Botia rostrata* (Mukherjee et al. 2022). The authors argue that this may be associated with a bioactive substance in the medicinal plants and phytoconstituents exhibit radical scavenging activity.

5. Conclusion

The dietary administration of myrtle and sage + PM showed positive effects on FCR as a result of feeding. The highest value in terms of lactic acid bacteria count was obtained from the groups fed with sage, while highest value in terms of total bacteria count was obtained in groups fed with myrtle + PM, sage and PM. It was also determined that the use of both plants alone and in combination with a PM was effective on intestinal morphology. The dietary administration of myrtle, PM, myrtle + PM improved disease resistance against *V. anguillarum*, and no mortalities or diseases were observed in the fish fed with sage + PM. The combination of probiotics and plant products could be used as an alternative disease control strategy in rainbow trout culture.

Data availability: Data are available on request due to privacy or other restrictions.

Ethics Committee Approval: The welfare of the fish was performed according to the ethical standards of the national guidelines approved by the Isparta Applied Sciences University Animal Care and Use Committee (decision number: 001, date: 24.09.2020).

Authorship Contributions: Concept: Ö.Ö., Ö.D., Design: Ö.Ö., Ö.D., M.H.K., Data Collection or Processing: Ö.Ö., M.H.K., Analysis or Interpretation: Ö.Ö., Ö.D., M.H.K., Literature Search: T.K.T., Z.B.S., B.I.D., Writing: Ö.Ö., Ö.D., M.H.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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