ARTICLE

Combined Effects of Dietary *Bacillus subtilis* and Trans-cinnamic Acid on Growth Performance, Whole Body Compositions, Digestive Enzymes and Intestinal bacteria in Rainbow Trout (*Oncorhynchus mykiss*)

Sevdan Yilmaz* Nergiz Çoban Sebahattin Ergun Murat Yigit Ekrem Şanver Çelik

1. Department of Aquaculture, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, 17100 Çanakkale, Turkey
2. Department of Marine Technology, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, 17100 Çanakkale, Turkey
3. Department of Marine Science, Faculty of Marine Sciences and Technology, Çanakkale Onsekiz Mart University, Çanakkale, 17100 Çanakkale, Turkey

ARTICLE INFO

**Article history**

Received: 1 February 2020
Accepted: 26 February 2020
Published Online: 31 March 2020

**Keywords:**
B. subtilis
Cinnamic acid
Organic acid
Probiotic
Oncorhynchus mykiss

**ABSTRACT**

In this study, the combined effects of dietary *Bacillus subtilis* (BS, 10^7 g/cfu) and different levels (0.025%, 0.050%, 0.075% and 0.150%) of trans-cinnamic acid (CA) on fish growth performance, whole body compositions, digestive enzymes, intestinal bacteria and internal organ index of rainbow trout (*Oncorhynchus mykiss*) were investigated. Six different experimental groups including control group (C), C+BS, 0.025%CA+BS, 0.050%CA+BS, 0.075CA+BS, 0.150%CA+BS) were established. According to the results obtained, growth performance, whole body compositions and digestive pH were not statistically significant among groups. Further, no significant differences were found between experimental groups in terms of the intestinal enzymes (trypsin, alkaline phosphatase and lipase) and gastric pepsin. Significantly higher levels of intestinal amylase were found in the control+BS, 0.025%CA+BS, 0.050%CA+BS, 0.075CA+BS, 0.150%CA+BS groups. Moreover, coliform and *Enterobacteriaceae* counts were highest in the control+B. subtilis and lowest in the 0.150% CA + B. subtilis groups.

1. Introduction

In order to supply the increasing demand for fish consumption in the world, aquaculture facilities face the challenge of intensive production of fish species in recirculating aquaculture systems, which eases the outbreak of diseases posing significant threat in terms of limitation on production amounts, lowering economic development in many countries [1].

The use of antibiotics is one of the most common method to control disease treatment in aquaculture facilities. However, the uncontrolled and unconscious use of antibiotics increases waste and accumulation in the environment and consequently negative impacts on terrestrial animals.
as well as humans can be seen in near future. In addition, intensive use of antibiotics increases the resistance of fish to pathogens in aquaculture facilities. These negative conditions caused by antibiotics led researchers to search for alternative environmentally friendly feed additives [2-4]. Alternative feed additives such as prebiotics, probiotics, algae, fungi, microalgae, enzymes, organic acids, myco-toxin binders, photogenic or phytoprotective compounds and yeasts, may not only increase growth performance of fish, but also increase the immune response of the fish and improve health condition [5].

Microbial balance and optimal pH levels in the digestive system eliminate pathogenic microorganisms. This is necessary to keep fish health at the desired level and to achieve expected production levels [6]. Probiotics balance microbial flora in the digestive tract by enhancing host health with complementary microorganisms such as bacteria, fungi and yeast [7-11]. They also improve feed quality and enzymatic activity in digestion and improve animal health and nutrition by activating the immune response. Probiotics are important alternative feed additives with potentially positive impacts in aquaculture facilities [14-17].

*Bacillus subtilis*, used in this study, breaks down pathogens and metabolizes nutrients appropriately and produces B-group vitamins including B7 (biotin) and B12 (kobalamint) [18-19]. In addition, bacterial spores of *B. subtilis* are easy to add to fish diets since they can remain in feed for a long time [20-21].

Trans-cinnamic acid (CA) is a natural polyphenolic organic acid derived from plants and known to have anti-fungal [22], anti-microbial [23], anti-oxidant [24], anti-tumor [25] and anti-inflammatory [26] effects. In addition, CA has been reported to have antimicrobial effect against bacteria such as *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Edwardsiella tarda* [27] and *Aeromonas sobria*, *Aeromonas salmonicida ATCC 33658*, *Listonella anguillarum* and *Yersinia ruckeri* [2,21].

Although CA is a suitable food additive due to its immune stimulating and antimicrobial effects [2,20,24,25], combined effects of dietary *Bacillus subtilis* and CA are limited with recent reports [43,48]. In this study, the combined effects of trans-cinnamic acid and *B. subtilis* on growth performance, nutrient composition, digestive enzymes and intestinal flora of rainbow trout (*Oncorhynchus mykiss*) were investigated.

2. Material and Methods

2.1 Fish and Experimental Design

Rainbow trout (*Oncorhynchus mykiss*) juveniles used in our study were obtained from a commercial trout farm (Keskin Alabalık Co., Bayramic-Canakkale). Before the start of the experiment, the fish were fed with commercial extruder diets (Anatolian Sea 50/4, Uğurlu Balık, Aydın-Turkey) for 2 weeks to adapt to the new conditions. *Bacillus subtilis* (0486C, *Bacillus subtilis* subsp. *spizizenii ATCC® 6633™* EZCFU) and trans-cinnamic acid (Al- drich W228826 trans-cinnamic acid natural, ≥99%, FCC, FG) used in the experiment were incorporated into the diets in fish oil at specified rates.

A total of 540 trout juveniles with a mean weight of 21.63±0.21 g were randomly allotted into 18-identical experimental tanks as 30 fish per tank (6 group × 3 replicate × 30 fish/tank). *B. subtilis* (BS) 10^7 cfu g\(^{-1}\) and cinnamic acid (CA) in ratios of 0.025%, 0.050%, 0.075% and 0.150% were added into the test diets. So the experimental groups were designed as 0% (control), 0% cinnamic acid + *B. subtilis* 10^7 cfu g\(^{-1}\), cinnamic acid 0.025%+*B. subtilis* 10^7 cfu g\(^{-1}\), cinnamic acid 0.050%+*B. subtilis* 10^7 cfu g\(^{-1}\), cinnamic acid 0.075%+*B. subtilis* 10^7 cfu g\(^{-1}\) and cinnamic acid 0.150%+*B. subtilis* 10^7 cfu g\(^{-1}\). At the end of the 60-day feeding experiment, fish growth performance, nutrient composition, total liver fat, internal organ indexes, intestinal and stomach enzymes, feed, stomach and intestinal pH amounts, and intestinal bacteria were analyzed.

2.2 Growth Performance and Feed Utilization

The following analyzes were used to calculate feed utilization [29]:

Relative growth rate, RGR (%) = final weight, g - initial weight, g / initial weight × 100

Specific growth rate, SGR (%Day\(^{-1}\)) = [Ln (final average weight, g) – Ln (initial average weight, g)] / trial days × 100

Feed conversion rate, FCR = feed consumption (g) / weight gain (g) × 100

2.3 Chemical Nutrient Analysis

2.3.1 Dry Matter Analysis

First, the internal organs of the fish were removed and fish were weighed. Fish were then dried in an oven at 70 °C until the constant weight was reached [29]. The samples were homogenized by grinding for protein, fat and ash analysis. Dry matter was calculated according to the following formula:

Dry matter (%) = 100 - [(sample weight + weight of foil pot) - (pot weight after drying)] / [(sample weight + weight of foil pot - weight of foil pot)] × 100

2.3.2 Protein Analysis

Kjeldahl method was used to determine the amount of
protein \[^{29}\]. Approximately 0.5 g of samples was taken into glass cylinder tubes and 1 catalyst tablet and 15 ml of sulfuric acid (H\(_2\)SO\(_4\)) were added. Protein digestion process was performed in BUCHI mark K-436 model infrared burning system. After cooling, the samples were taken to BUCHI mark K-350 model distillation system. Then, it was titrated with 0.1 moles of Hydrochloric acid (HCl). The percentage of protein was calculated according to the following formula:

\[
\text{Crude protein \%} = \frac{\text{(discharge at titration - blind sample)} \times 0.1}{\text{Normality of HCl solution}} \times 14.007 \left(\text{Milli-equivalent weight of nitrogen}\right) \times 6.25(\text{Factor}) / \text{sample weight} \times 100
\]

2.3.3 Fat Analysis

In fat analysis, 0.5 g of fish and feed samples and 0.25 g of liver samples were weighed in test tubes with lids and methanol/chloroform mixture was added. The samples were kept in the dark for 1 night. Then the samples were filtered and taken to the first weighed test tubes. methanol/chloroform was removed in a 40 °C water bath with a nitrogen evaporator. Afterwards, the tubes were taken into the desiccator and weighed \[^{30}\]. The amount of crude fat was calculated according to the following formula:

\[
\text{Crude fat amount \%} = \frac{\text{weight change of glass bubble (g)}}{\text{sample weight (g)}} \times 100
\]

2.3.4 Ash Analysis

For the analyses of ash content, 0.5 g of samples were taken and put into pre-tared porcelain crucibles. Then, the crucibles were fired in the incinerator at 525 °C for 12 hours \[^{29}\]. The ash content was calculated according to the following formula:

\[
\text{Crude ash content \%} = \frac{\text{weight change of porcelain crucible (g)}}{\text{sample weight (g)}} \times 100
\]

2.4 Biometric Indices

Biometric indices were calculated using the following equations:

- Visceral fat index (VFI) = \[\frac{\text{wet weight of visceral fat (g)}}{\text{wet body weight (g)}} - \text{wet weight of visceral fat (g)}\] \times 100

- Hepatosomatic index (HSI) = \[\frac{\text{wet weight of liver (g)}}{\text{wet body weight (g)}} - \text{wet weight of liver (g)}\] \times 100

- Viscerosomatic index (VSI) = \[\frac{\text{wet weight of viscera and associated fat (g)}}{\text{wet body weight (g)}} - \text{wet weight of viscera and associated fat (g)}\] \times 100

- Bile–somatic index (BSI) = \[\frac{\text{wet weight of bile (g)}}{\text{wet body weight (g)}} - \text{wet weight of bile (g)}\] \times 100

Spleen–somatic index (SSI) = \[\frac{\text{wet weight of spleen (g)}}{\text{wet body weight (g)}} - \text{wet weight of spleen (g)}\] \times 100

2.5 Intestinal Bacteria and pH Analysis

Total bacteria, total yeast, mold, coliform and lactic acid bacteria were counted in order to determine the effects of cinnamic acid and Bacillus subtilis on intestinal bacteria. For 1 g intestine sample, 3 fish were taken from each tank and the anterior intestine was combined. Sterile PBS was added to the intestinal samples at a rate of 9 times and homogenized with glass homogenizers. Then, dilution was applied at a rate of 9 times. The counts were applied by smear and pouring plate methods \[^{31}\] and methods previously reported in trout were used \[^{32-34}\].

Microbiological analyses were performed as (a) determination of total heterotrophic and mesophilic aerobic counts on Tryptic Soy Agar (TSA; Merck) at 22 °C for 36 h, and 37°C for 24 h respectively; (b) determination of total yeast and mold counts on Potato Dextrose Agar (PTA; Sigma-Aldrich) at 22 °C for 120 h; (c) determination of total coliform counts on MacConkey Agar (MA; Merck) at 37°C for 24 h; (d) determination of total Enterobacteriaceae counts on Violet Red Bile Glucose Agar (VRBG; Merck) at 37°C for 24 h; and (e) determination of total Lactobacillus counts on de Man, Rogosa, and Sharpe Agar (MRSA) at 37°C for 120 h. Bacterial counts were expressed as log CFU g\(^{-1}\) of wet matter.

The pH values were measured with tabletop pH meter in fish feed, intestine and stomach (HI 2221).

2.6 Intestinal and Stomach Enzymes

Prior to analysis, stomach and intestinal samples were homogenized in cold pure water and was centrifuged at 30000 rcf for 30 minutes at 4 °C. The supernatants were stored at -80 °C until use in the assays. Protein ratio for each sample was determined by Bradford \[^{35}\] method analyzes. The concentration of trypsin \[^{36}\], amylase \[^{37}\], lipase \[^{38}\], alkaline phosphatase and pepsin \[^{39}\] was estimated according to the methods previously conducted and described in our laboratory \[^{32-34}\].

3. Results

3.1 Growth Performance

At the end of the experiment, average initial weight (IW), average final weight (FW), relative growth rate (RGR), feed conversion rate (FCR) and specific growth rate (SGR) results are given in Table 1. According to the results of the study, no statistical significance in growth performance was observed in rainbow trout (O. mykiss) juvenils fed ex-
3.2 Whole Body Composition and Liver Fat

At the end of the experiment, nutritional composition of *B. subtilis* and cinnamic acid + *B. subtilis* groups were evaluated and presented in Table 2. According to the data obtained, it was found that there were no statistically effects on dry matter, protein, fat and ash and liver fat (p>0.05).

3.3 Intestinal Bacteria

At the end of the experiment, the dietary incorporation of *B. subtilis* and cinnamic acid + *B. subtilis*, did not show any significant influence on the intestinal bacteria (p>0.05). However, it was observed that *B. subtilis* was isolated back in the intestines in all groups with the addition of *B. subtilis* (Table 3). When mesophilic bacteria, coliform and Enterobacteriaceae data were evaluated, the highest values were found in the control + *B. subtilis* group, while the lowest values were found in the group containing 0.150% CA + *B. subtilis*.

3.4 Feed, Stomach and Intestinal pH

The effects of dietary incorporation of *B. subtilis* and cinnamic acid + *B. subtilis* on pH values of the feed, intestine and stomach are shown in Table 4. Stomach and intestinal pH values were similar in all groups at day 60 (p>0.05).

| Table 1. Growth performance and feed evaluation of rainbow trout juvenil at the end of the trial |
|---|---|---|---|---|---|---|---|
| | Control | Control+ *B. subtilis* | %0.025 CA+*B. subtilis* | %0.050 CA+*B. subtilis* | %0.075 CA+*B. subtilis* | %0.150 CA+*B. subtilis* |
| IW (g) | 21.10±2.96 | 21.53±3.41 | 21.81±3.38 | 21.81±3.38 | 21.54±3.34 | 22.00±3.30 |
| FW (g) | 55.52±4.30 | 53.55±4.28 | 55.16±3.80 | 54.41±3.66 | 55.74±3.75 | 56.17±3.07 |
| RGR (%) | 168.83±20.25 | 156.54±24.94 | 157.88±27.30 | 157.88±27.35 | 167.46±28.19 | 164.51±30.81 |
| FCR | 1.16±0.10 | 1.21±0.05 | 1.17±0.03 | 1.20±0.03 | 1.14±0.03 | 1.16±0.02 |
| SGR (% day^-1) | 1.64±0.12 | 1.56±0.16 | 1.58±0.17 | 1.56±0.17 | 1.62±0.17 | 1.60±0.19 |

| Table 2. Biochemical composition of fish meat and liver fat (excluding internal organs) |
|---|---|---|---|---|---|---|
| | Control | Control+ *B. subtilis* | %0.025 CA+*B. subtilis* | %0.050 CA+*B. subtilis* | %0.075 CA+*B. subtilis* | %0.150 CA+*B. subtilis* |
| Dry Matter* (%) | 29.23±0.24 | 29.10±0.20 | 30.00±0.52 | 29.10±0.20 | 30.00±0.52 | 29.10±0.20 |
| Protein (%) | 16.53±0.26 | 16.23±0.26 | 16.53±0.26 | 16.23±0.26 | 16.53±0.26 | 16.23±0.26 |
| Fat (%) | 9.76±0.30 | 9.79±0.27 | 9.76±0.30 | 9.79±0.27 | 9.76±0.30 | 9.79±0.27 |
| Ash (%) | 2.73±0.07 | 2.71±0.04 | 2.73±0.07 | 2.71±0.04 | 2.73±0.07 | 2.71±0.04 |
| Liver Fat (%) | 6.43±0.19 | 5.88±0.45 | 5.57±0.31 | 5.16±0.25 | 5.94±0.42 | 5.86±0.43 |

Note: *The percentages of protein, fat and ash results are expressed as % in dry matter.

| Table 3. Total counts of bacterial groups and yeasts and Moulds (log CFU g^-1) in the intestines |
|---|---|---|---|---|---|---|
| | Control | Control+ *B. subtilis* | %0.025 CA+*B. subtilis* | %0.050 CA+*B. subtilis* | %0.075 CA+*B. subtilis* | %0.150 CA+*B. subtilis* |
| *Total Heterotrophic Aerobic Bacteria* | 5.49±0.57 | 6.73±0.15 | 5.96±0.67 | 4.90±0.42 | 5.86±0.72 | 5.49±0.29 |
| *Mesophilic Bacteria* | 3.61±0.34 | 4.93±0.39 | 3.31±0.31 | 3.47±0.62 | 3.47±0.77 | 2.74±0.02 |
| *B. subtilis* | 5.49±0.26 | 5.29±0.06 | 5.49±0.12 | 5.80±0.49 | 5.53±0.17 |
| Yeast and Mould | 3.78±0.77 | 3.57±0.23 | 4.12±0.10 | 4.06±0.39 | 4.47±0.68 | 4.09±0.36 |
| Coliform | 2.49±1.15 | 3.73±0.37 | 1.56±0.98 | 1.53±1.09 | 2.67±1.19 | 0.54±0.16 |
| Enterobacteriaceae | 2.83±1.20 | 4.39±0.16 | 2.29±0.95 | 2.03±1.49 | 3.33±1.52 | 1.20±0.75 |
| Lactic Acid Bacteria | 0.36±0.06 | 0.74±0.13 | 0.56±0.04 | 0.52±0.14 | 0.57±0.13 | 0.65±0.17 |

Note: * While the number of mesophile bacteria and total heterotrophic aerobic bacteria were calculated in the groups containing *B. subtilis*, the amount of *B. subtilis* was not added to the count since it was given separately.
3.5 Intestinal and Stomach Enzyme

At the end of the experiment, trypsin, amylase, lipase, alkaline phosphatase and pepsin values of intestinal and gastric enzymes of B. subtilis and cinnamic acid + B. subtilis groups were analyzed and findings are shown in Table 5. There was no statistically significant difference between the groups of intestinal enzymes trypsin, alkaline phosphatase, lipase and gastric pepsin (p>0.05). However, it was found that the amount of intestinal amylase was higher in the groups control+B.subtilis, 0.025%CA+B.subtilis, 0.050%CA+B.subtilis and 0.075%CA+B.subtilis compared to the control and 0.150%CA+B.subtilis groups (p<0.05).

3.6 Internal Organ Index

Visceromatic index (VSI), hepatosomatic index (HSI), visceral fat somatic index (VFSI), bile somatic index (BSI), spleen somatic index (SSI) and heart somatic index (HSI) values of the internal organ indexes of groups fed diets incorporated with B. subtilis and CA+B.subtilis are given in Table 6. At the end of the study, it was found that VSI, VFSI and GSI values of the experimental groups were similar to the control group (p>0.05). HSI ratio was found to be lower in group 0.075%CA+B.subtilis than the control and the 0.050%CA+B.subtilis groups (p<0.05). SSI ratio was found to be statistically higher in group 0.150%CA+B.subtilis than all other groups (p<0.05). HSI values were found to be statistically lower in group 0.075%CA+B.subtilis than the control group (p<0.05).

4. Discussion

The combination of probiotic and organic acids at dietary incorporation levels tested in this study, did not influence growth performance of rainbow trout. This was in agree-

**Table 4.** Changes in pH values of feed, stomach and intestines

| Experiment Groups | Feed pH | Stomach pH | Intestine pH |
|-------------------|---------|------------|-------------|
| Control           | 5.90    | 6.81±0.04  | 7.07±0.01  |
| Control+ B.subtilis | 5.89    | 6.88±0.03  | 7.07±0.01  |
| %0.025 CA+B.subtilis | 5.86    | 6.92±0.02  | 7.06±0.02  |
| %0.050 CA+B.subtilis | 5.83    | 6.87±0.04  | 7.05±0.02  |
| %0.075 CA+B.subtilis | 5.75    | 6.91±0.02  | 7.04±0.01  |
| %0.150 CA+B.subtilis | 5.74    | 6.93±0.03  | 7.08±0.01  |

**Table 5.** Changes of trypsin, amylase, lipase and alkaline phosphatase in the intestine and pepsin enzymes in the stomach

| Experiment Groups | Trypsin (U/mg protein/min) | Amylase (mU/mg protein) | Lipase (uMol/mg protein/min) | Alkaline phosphatase (U/mg protein/min) | Pepsin (U/mg protein/min) |
|-------------------|---------------------------|-------------------------|-----------------------------|----------------------------------------|--------------------------|
| Control           | 1.66±0.26                 | 57.48±6.71              | 0.28±0.02                   | 0.23±0.03                              | 34.59±6.34               |
| Control+ B.subtilis | 1.57±0.16                 | 249.79±33.60            | 0.22±0.02                   | 0.21±0.03                              | 30.12±4.82               |
| %0.025 CA+B.subtilis | 1.66±0.15                 | 207.65±21.67            | 0.22±0.01                   | 0.26±0.03                              | 31.20±3.08               |
| %0.050 CA+B.subtilis | 1.43±0.14                 | 250.62±26.65            | 0.20±0.02                   | 0.17±0.02                              | 39.46±4.31               |
| %0.075 CA+B.subtilis | 2.02±0.19                 | 190.86±20.28            | 0.26±0.05                   | 0.20±0.03                              | 32.29±3.65               |
| %0.150 CA+B.subtilis | 1.53±0.12                 | 62.83±6.65              | 0.20±0.02                   | 0.24±0.03                              | 26.74±2.24               |

**Table 6.** Changes in internal organ indexes

| Parameters | Control | Control+ B.subtilis | %0.025 CA+B.subtilis | %0.050 CA+B.subtilis | %0.075 CA+B.subtilis | %0.150 CA+B.subtilis |
|------------|---------|---------------------|----------------------|----------------------|----------------------|----------------------|
| VSI        | 15.31±0.63<sup>a</sup> | 16.21±0.59<sup>a</sup> | 14.35±0.50<sup>a</sup> | 17.07±0.23<sup>a</sup> | 14.62±0.54<sup>a</sup> | 15.68±0.86<sup>a</sup> |
| HSI        | 1.56±0.08<sup>b</sup>  | 1.42±0.05<sup>b</sup>  | 1.34±0.06<sup>b</sup>  | 1.59±0.08<sup>b</sup>  | 1.25±0.05<sup>b</sup>  | 1.46±0.06<sup>b</sup>  |
| VFSI       | 4.00±1.30<sup>a</sup>  | 4.31±2.00<sup>a</sup>  | 3.86±2.3<sup>a</sup>    | 4.88±1.0<sup>a</sup>   | 4.50±0.31<sup>a</sup>  | 4.12±0.29<sup>a</sup>  |
| BSI        | 0.21±0.02<sup>a</sup>  | 0.18±0.03<sup>a</sup>  | 0.13±0.01<sup>a</sup>   | 0.20±0.03<sup>a</sup>  | 0.14±0.01<sup>a</sup>  | 0.12±0.01<sup>a</sup>  |
| SSI        | 0.34±0.02<sup>a</sup>  | 0.34±0.01<sup>a</sup>  | 0.33±0.03<sup>a</sup>   | 0.36±0.04<sup>a</sup>  | 0.33±0.01<sup>a</sup>  | 0.47±0.02<sup>a</sup>  |
| HSI        | 0.23±0.01<sup>a</sup>  | 0.21±0.01<sup>a</sup>  | 0.19±0.02<sup>a</sup>   | 0.22±0.02<sup>a</sup>  | 0.17±0.02<sup>a</sup>  | 0.22±0.01<sup>a</sup>  |
ment with a recent study on rainbow trout (Onchorhynchus mykiss), where dietary inclusion of Bacillus subtilis did not show any impact on fish growth.[18] No significant differences in terms of fish growth were found in flounder fed diets incorporated with a mixture of organic acids.[40]. In another study, giant grouper Epinephelus lanceolatus demonstrated the lowest growth performance when fish was fed a diet with 1% lactase addition compared to the other test groups.[41]. In contrast to these reports, Hassaan et al.[9], reported significantly higher values for weight gain (WG), specific growth rate (SGR) and Feed conversion ratio (FCR) on nile tilapia (Oreochromis niloticus), fed a combination of Bacillus subtilis and malic acid compared to the control group. Further, Nesara et al.[42] also found higher growth rate in Labeo rohita, fed diets incorporated with Lactobacillus plantarum and citric acid combinations over the control group without dietary treatments. The probiotic combination of B. subtilis and B. licheniformis increased growth rate of rainbow trout (O. mykiss) [3], and experimental diets with Bacillus subtilis addition also increased growth rate in red sea bream (Pagrus major) [14].

Similar to our findings in this study, Yilmaz et al.,[43] did not find significant change in dry matter, protein, fat and ash contents of rainbow trout fed diets with cinnamic acid incorporation. Microbial diversity affects the digestive system and probiotic application plays an important role in regulating and functioning of this system.[43]. Earlier studies have also reported that B. subtilis may support growth performance and survival of animals and humans.[44-45-46]. In this study, however, experimental treatment groups with B. subtilis did not show significant changes on the intestinal bacteria, which is in agreement with the findings of Wu et al.,[44], who investigated the effects of B. subtilis on the intestinal bacteria and found similar counts for the total aerobic and facultative anaerobic bacteria in all experimental groups including the control.

Organic acids, mineral absorption, nutrient digestion and accumulation of H+ ions reduce the level of pH in the digestive tract and can positively affect growth performance.[43-47]. In our study, it was observed that pH levels in the digestive system did not cause any changes between experimental groups. However, Yilmaz et al.,[43] reported that cinnamic acid supplements decreased the pH levels of the stomach and intestines 4 hours after feeding. Culture media containing cinnamic acid did not show antimicrobial effect on B. subtilis.[43] This shows that cinnamic acid provides acidic condition to improve B. subtilis in the digestive tract. Therefore, low doses of B. subtilis and cinnamic acid mixtures are encouraged to be investigated.

Digestive enzyme activities are important data for digestive capacity and growth performance.[44,45,46]. In our study, no statistically significant differences were found between trypsin, alkaline phosphatase, lipase and pepsin groups from intestinal and stomach enzymes. Similarly, in a study on rainbow trout, amylase, lipase and trypsin values did not differ between experimental groups.[43]. However, it was observed that cinnamic acid supplementation increased stomach pepsin activity.[43]. In the study with Ctenopharyngodon idella, it was observed that the addition of B. subtilis Ch9 increased amylase and lipase activity in the intestine.[44]. Another study investigating the effects of malic acid in fish diets, reported increased levels for gastric pepsin activity and growth performance in tilapia (Oreochromis niloticus).[51].

Enterobacteriaceae is usually found in the gastrointestinal tract of fish and its presence in fish farming can cause serious problems for human health.[52]. Degree of contamination of coliform bacteria gives information about fish quality.[53]. So, according to the studies, contamination of enteric bacteria in the intestinal micflora[44] of human or animal may cause food spoilage.[55]. Faecal colloforms in fish also show the level of pollution in the environment, because coliforms are not found in a normal flora of fish.[56]. In our study, the coliform and Enterobacteriaceae counts were highest in the “control+B. subtilis” and lowest in the “0.150% CA + B. subtilis” groups. In combination with B. subtilis and high doses of cinnamic acid, the bacterial count is reduced. So, cinnamic acid can suppress harmful bacteria when used within appropriate doses.

Internal organ indexes may increase or decrease in case of unhealthy conditions.[57]. Spleen, an important organ in fish, is the place where erythrocytes and neutrophils are produced and matured.[58-59]. Spleen has an important role in the immune response in fish.[58-60]. In our study, SSI value increased in the group containing 0.150% CA+B. subtilis. In an earlier study conducted on the effect of herbal supplement in sea bass diets, SSI values in experimental fish were increased compared to the control group.[58]. In another study, the spleen size was found to be positively influenced by disease resistance.[61]. SSI value can provide information about fish health and immunity. HSI values were lowest in the group containing 0.075% CA+B. subtilis compared to the control+B. subtilis. Similarly, HSI values were reduced in the study conducted on sea bass.[58]. In our study, HSI values were reduced in the study conducted on sea bass.[58]. In another study, the spleen size was found to be positively influenced by disease resistance.[61]. SSI value can provide information about fish health and immunity. HSI values were lowest in the group containing 0.075% CA+B. subtilis compared to the control+B. subtilis. Similarly, HSI values were reduced in the study conducted on sea bass.[58], this could be linked to some improvements in the organs.

In our study, the combination of B. subtilis and cinnamic acid did not show statistically significant effects on growth performance, whole body composition values, and gastrointestinal system pH values. However, according to internal organ index and intestinal bacteria results, we have observed that it reduces coliform and Enterobacter-
riaceae counts when used at appropriate doses. Also, organic acid and probiotics may be used as additives for the healing of internal organs.

Experimental conditions such as feeding periods, type and size of fish, different organic acid or probiotics used and dosage ranges could be explained as reasons for the differences between different studies. Therefore, further investigations on different conditions are encouraged to find out best results.

Acknowledgments

The present study was conducted as a partial fulfilment of the PhD thesis of the first author and summarized from a part of the thesis supported by TUBITAK (Scientific and Technological Research Council of Turkey) with the Project Number of 1130364.

References

[1] Park, Y., Lee, S., Hong, J., Kim, D., Moniruzzaman, M., Bai, S.C.. Use of probiotics to enhance growth, stimulate immunity and confer disease resistance to Aeromonas salmonica in rainbow trout (Oncorhynchus mykiss), Aquaculture Research, 2016: 1–11.

[2] Yılmaz, S., Ergün, S.. Trans-cinnamic acid application for rainbow trout (Oncorhynchus mykiss): I. Effects on haematological, serum biochemical, non-specific immune and head kidney gene expression responses, Fish and Shellfish Immunology, 2018, 78: 140–157.

[3] Chi, C., Giri, S.S., Jun, J.W., Yun, S., Kim, H.J., Kim, S.G., Park, S.C.. Immune response of the bay scallop, Argopecten irradians, after exposure to the algicde palmitoleic acid, Fish Shellfish Immunol., 2016, 57: 371–378.

[4] Nayak, S., Probiotics and immunity: a fish perspective, Fish Shellfish Immunol., 2010, 29 (1): 2–14.

[5] Bharathi, S., Antony, C., Cbt, R., Arumugam, U., Ahilan, B., Aanand, S.. Functional feed additives used in fish feeds, International Journal of Fisheries and Aquatic Studies, 2019, 7(3): 44-52.

[6] Hassaan, M., Soltan, M., Jarmolowicz, S., Abdo, H.. Combined effects of dietary malic acid and Bacillus subtilis on growth, gut microbiota and blood parameters of Nile tilapia (Oreochromis niloticus), Aquaculture Nutrition, 2018, 24: 83–93. https://doi.org/10.1111/anu.12536

[7] Kamgar, M., Ghane M.. Evaluation of Bacillus subtilis effect as probiotic on hematological parameters of rainbow trout, Oncorhynchus mykiss (Walbaum) following experimental infection with Streptococcus iniae, Journal of fisheries and aquatic science, 2012, 7(6): 422-430.

[8] Vivas, J., Riano, J., Carracedo, B., Razquin, B. E., Lopez-Fierro, P., Naharro, G. and Villena, A. J. The auxotrophic aroA mutant of A. hydrophila as alive attenuated vaccine against A. salmonicida infections in rainbow trout, Fish & Shellfish Immunology, 2004, 16: 193-206.

[9] Bagheri, T., Hedayati, S. A., Yavari, V., Alizade, M., Farzianfar, A.. Growth, Survival and Gut Microbial Load of rainbow trout, Onchorhynchus mykiss (Walbaum) Fry Given Diet Supplemented with Probiotic during the Two Months of First Feeding, Journal of Fisheries and Aquatic Science, 2008, 8: 43-48.

[10] Hung, A. T. Y., Su, T. M., Liao, C. W., Lu, J. J.. Effect of Probiotic Combination fermented Soybean Meal on Growth Performance, Lipid Metabolism and Immunological Response of Growing-Finishing Pigs, Asian Journal of Animal and Veterinary Advances, 2008, 3: 431-436.

[11] Zhou, Q., Li K., Jun, X., Bo, L.. Role and Functions of beneficial microorganisms in sustainable aquaculture, Bioresource Technology, 2009, 100: 3780-3786.

[12] Son, V. M., Chang, C. C., Wu, M. C., Guu, Y. K., Chiu, C. H., Cheng, W.. Dietary administration of probiotic, Lactobacillus plantarum, enhanced the growth, innate immune responses and disease resistance of grouper Epinephelus coioides, Fish & Shellfish immunology, 2009, 26: 691-698.

[13] Agouz, H. M., Anwer. W.. Effect of Biogen® and Myco-Ad® on the Growth Performance of Common Carp (Cyprinus carpio) Fed a Mycotoxin Contaminated Aquafeed, Journal of Fisheries and Aquatic Science, 2011, 6 (3): 334–345.

[14] Zaineldin, A.I., Hegazi, S., Koshio, S., Ishikawa, M., Bakr, A., El-Keredy, A.M.S., Dawood, M.A.O., Dossou, S., Wang, W., Yukun, Z.. Bacillus subtilis as probiotic candidate for red sea bream: Growth performance, oxidative status, and immune response traits, Fish and Shellfish Immunology, 2018, 79: 303–312.

[15] Dawood, M.A., Koshio, S., Ishikawa, M., El-Sabagh, M., Esteban, M.A., Zaineldin, A.I.. Probiotics as an environment-friendly approach to enhance red sea bream, Pagrus major growth, immune response and oxidative status, Fish Shellfish Immunol., 2016, 57: 170–178.

[16] Adel, M., Yeganeh, S., Dawood, M.A.O., Safari, R., Radhakrishnan, S.. Effects of Pediococcus pentosaceus supplementation on growth performance, intestinal microflora and disease resistance of white shrimp, Litopenaeus vannamei, Aquacult. Nutr., 2017, 23(6): 1401–1409.

[17] Abdelkhalek, N.K., Eissa, I.A., Ahmed, E., Kilany,
O.E., El-Adl, M., Dawood, M.A.O., Hassan, A.M., Abdel-Daim, M.M.. Protective role of dietary Spirulina platensis against diazinon-induced Oxidative damage in Nile tilapia; Oreochromis niloticus, Environ. Toxicol. Pharmacol., 2017, 54: 99–104.

[18] Sahraei, F., Ahari, H., Kakoolaki, S.. Effect of Bacillus subtilis as a probiotic on protein, lipid content, and trypsin and chymotrypsin enzymes in rainbow trout biometry (Oncorhynchus mykiss), Aquaculture International, 2019, 27: 141–153.

[19] Mombelli B., Gismondo M.R.. The use of probiotics in medical practice, Int J Antimicrob Agents, 2000, 16: 531–536.

[20] Yılmaz, S., Ergün, S., Yiğit, M., Çelik, E.Ş.. Effect of combination of dietary Bacillus subtilis and trans-cinnamic acid on innate immune responses and resistance of rainbow trout, Oncorhynchus mykiss to Yersinia ruckeri, Aquaculture Research., 2019a, 00: 1–14.

[21] Yılmaz, S.. The Effect of Dietary Cinnamic Acid or Bacillus subtilis on Growth Performance and Immunological Parameters in Rainbow Trout, Department of Aquaculture, Çanakkale Onsekiz Mart University, Graduate School of Natural and Applied Sciences, Türkiye, 2017: 210.

[22] Said, S., Neves, F.M., Griffiths, A.J.F.. Cinnamic acid inhibits the growth of the fungus Neurospora crassa, but is eliminated as acetophenone, Int. Biodeterior. Biodegrad, 2004, 54(1): 1–6.

[23] Sova, M.. Antioxidant and antimicrobial activities of cinnamic acid derivatives, Mini Rev. Med. Chem., 2012, 12(8): 749–767.

[24] Pontiki, E., Hadjipavlou-Litina, D., Litinas, K., Geromichalos, G.. Novel cinnamic acid derivatives as antioxidant and anticancer agents: design, synthesis and modeling studies, Molecules, 2014, 19(7): 9655–9674.

[25] Liu, L., Hudgins, W.R., Shack, S., Yin, M.Q., Samid, D.. Cinnamic acid - a natural product with potential use in cancer intervention, Int. J Canc., 1995, 62(3): 345–350.

[26] Fernandez, M., Saenz, M., Garcia, M.. Natural Products: anti-inflammatory activity in rats and mice of phenolic acids isolated from Scrophularia frutescens, J. Pharm. Pharmacol., 1998, 50(10): 1183–1186.

[27] Prasad, V.G.N.V., Swamy, P.L., Rao, T.S. and Rao, G.S. (2014) Antibacterial synergy between quercetin and polyphenolic acids against bacterial pathogens of fish, Asian Pac. J. Trop. Dis., 4, S326–S329.

[28] Yılmaz, S., Ergün S.. Chickweed (Stellaria media) Leaf Meal as a Feed Ingredient for Tilapia (Oreochromis mossambicus), Journal of Applied Aquaculture, 2013, 25(4): 329-336.

[29] AOAC. Official methods of analysis of AOAC International, AOAC Int., Gaithersburg, MD, 1998.

[30] Folch, J., Lees, M., Sloane-Stanley, G. H.. A simple method for the isolation and purification of total lipids from animal tissues, J biol chem, 1957, 226(1): 497-509.

[31] Anonymous. Merck Gıda Mikrobiyolojisi Uygulamaları. Ed: A. K. Halkman, Başak Matbaacılık Ltd. Şti., Ankara, 2005.

[32] Merrifield, D. L., Dimitroglou, A., Bradley, G., Baker, R. T. M., Davies, S. J.. Probiotic applications for rainbow trout (Oncorhynchus mykiss Walbaum) I. Effects on growth performance, feed utilization, intestinal bacteria and related health criteria, Aquaculture Nutrition, 2010, 16(5): 504-510.

[33] Giannenas, I., Triantafillou, E., Stavrakakis, S., Margaroni, M., Mavridis, S., Steiner, T., Karagouni, E.. Assessment of dietary supplementation with carvacrol or thymol containing feed additives on performance, intestinal bacteria and antioxidant status of rainbow trout (Oncorhynchus mykiss), Aquaculture, 2012, 350: 26-32.

[34] Šyvokienė, J. and Vosylienė, M.Z.. Impact of copper and zinc mixture on bacterial flora of digestive tract of rainbow trout (Oncorhynchus mykiss), Journal of environmental engineering and landscape management, 2013, 21(4): 288–295.

[35] Bradford, M.M.. A rapid and sensitive method for the quantization of protein utilizing the principle of dye-protein binding, Analytical Biochemistry, 1976, 72: 248-254.

[36] Faulk, C. K., Benninghoff, A. D., Holt, G. J.. Ontogenic, dietary, and phylogenetic effects, Physiological and Biochemical Zoology, 2004, 77(5): 789-804.

[37] Jiang, H., Wang, X.. Time-dependent nanogel aggregation for naked-eye assays of α-amylase activity, Analyst, 2012, 137(11): 2582-2587.

[38] German, D. P., Horn, M. H., Gawlicka, A.. Digestive enzyme activities in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects, Physiological and Biochemical Zoology, 2004, 77(5): 789-804.

[39] Worthington, V.. Worthington Enzyme Manual. New Jersey, US. 1993: 399.

[40] Katya, K., Park, G., Bharadwaj, A.S., Browdy, C.L., Vazquez-Anon, M., Bai, S.C.. Organic acids blend as dietary antibiotic replacer in marine fish olive flounder, Paralichthys olivaceus, Aquaculture Research, 2018, 49: 2861-2868.
[41] Lin Y., Cheng M.. Effects of dietary organic acid supplementation on the growth, nutrient digestibility and intestinal histology of the giant grouper Epinephelus lanceolatus fed a diet with soybean meal, Aquaculture, 2017, 469: 106–111.

[42] Nesara, K., Jayaraj, E., Amoga, K., Sandeep, C.. Effects of dietary probiotic and organic acid alone and in combination on the growth performance of Indian major carp, Labeo rohita, Journal of Experimental Zoology, 2018, 21: 805–812.

[43] Yilmaz, S., Ergun, S., Çelik, E.Ş., Yigit, M., Bayizit, C., Dietary trans-cinnamic acid application for rainbow trout (Oncorhynchus mykiss): II. Effect on antioxidant status, digestive enzyme, blood biochemistry and liver antioxidant gene expression responses, Aquaculture Nutrition, 2019b. https://doi.org/10.1111/anu.12935

[44] Wu, Z. X., Feng, X., Xie, L. L., Peng, X. Y., Yuan, J., Chen, X. X.. Effect of probiotic Bacillus subtilis Ch9 for grass carp, Ctenopharyngodon idella (Valenciennes, 1844), on growth performance, digestive enzyme activities and intestinal microflora, Journal of Applied Ichthyology, 2012, 28(5): 721–727. https://doi.org/10.1111/j.1439-0426.2012.01968.x

[45] Hoa, N. T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P., Ammendola, S., Ricca, E., Cutting, S. M. Characterization of bacillus species used for oral bacteriotherapy and bacteriophylaxis of gastrointestinal disorders, Appl. Environ. Microbiol., 2000, 66: 5241–5247.

[46] McCarthy, J., OMahony, L., OCallaghan, L., Sheil, B., Vaughan, E. E., Fitzsimons, N., Fitzgibbon, J., OSullivan, G. C., Kiely, B., Collins, J. K., Shanahan, F. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance, Gut, 2003, 52: 975–980.

[47] Ng, W. K., Koh, C. B.. The utilization and mode of action of organic acids in the feeds of cultured aquatic animals, Reviews in Aquaculture, 2017, 9(4): 342–368. https://doi.org/10.1002/raq.12141

[48] Yilmaz, S., Sova, M., Ergün, S.. Antimicrobial activity of trans cinnamic acid and commonly used antibiotics against important fish pathogens and non-pathogenic isolates, Journal of Applied Microbiology, 2018, 125(6): 1714–1727. https://doi.org/10.1111/jam.14097

[49] Ueberschar, B.. The use of trypptic enzyme activity measurement as a nutritional condition index: laboratory calibration data and field application, ICES Mar. Sci. Symp., 1995, 201: 119–129.

[50] Sузer, C., Çoban, D., Okan, K. H., Saka, S., Firat, K., Oktugcuoglu, O., Kucukkars, H.. Lactobacillus spp. bacteria as probiotics in gilthead sea bream (Sparus aurata, L.) larvae: effects on growth performance and digestive enzyme activities, Aquaculture, 2008, 280: 140–145.

[51] Chen, Y. J., Luo, L., Zhang, G. Z., Li, Z., Bai, F. J., Shi, Y. Q., Yang, H. S.. Effect of dietary L-malic acid supplementation on growth, feed utilization and digestive function of juvenile GIFT tilapia Oreochromis niloticus (Linnaeus, 1758), Journal of Applied Ichthyology, 2016, 32(6): 1118–1123. https://doi.org/10.1111/jai.13119

[52] Oliveira, R.V., Oliveira, M.C., Pelli, A.. Disease Infection by Enterobacteriaceae Family in Fishes: A Review, J Microbiol Exp, 2017, 4(5): 00128.

[53] Al-Harbi, A.H.. Faecal coliforms in pond water, sediments and hybrid tilapia Oreochromis niloticus ×Oreochromis aureus in Saudi Arabia, Aquaculture Research, 2003, 34: 517-524.

[54] Kaneko, S.. Microbiological study of fresh fish, NewFood Industry, 1971, 13: 76-80.

[55] Geldreich, E.E., Clarke, N.A.. Bacterial pollution indicators in the intestinal tract of freshwater fish, Applied Microbiology, 1966, 14: 429-437.

[56] Cohen, J., Shuval, H.I.. Coliforms, fecal coliform and fecal streptococci as indicators of water pollution, Water Soil Pollution, 1973, 2: 85-95.

[57] Hadidi, S., Glenney, G. W., Welch, T. J., Silverstein, J. T., Wiens, G. D.. Spleen size predicts resistance of Rainbow Trout to Flavobacterium psychrophilum challenge. Journal of Immunology, 2008, 180: 4156–4165.

[58] Yilmaz, S., Ergün S., Çelik, E.Ş.. Effect of Dietary Herbal Supplements on Some Physiological Conditions of Sea Bass Dicentrarchus labrax, Journal of Aquatic Animal Health, 2013, 25: 98–103.

[59] Anderson, D. P.. Fish immunology. T.F.H. Publications, Neptune City, New Jersey, 1974.

[60] Kumaran, S., Deivasigamani, B., Alagappan, K. M., Saktivel, M.. Infection and immunization trials of Asian Seabass (Lates calcarifer) against fish pathogen Vibrio anguillarum, Journal of Environmental Biology, 2010, 31: 539–541.

[61] Wiens, G. D., Vallejo R. L.. Temporal and pathogen-load dependent changes in Rainbow Trout (Oncorhynchus mykiss) immune response traits following challenge with biotype 2 Yersinia ruckeri, Fish and Shellfish Immunology, 2010, 29: 639–647.

[62] Yilmaz, S., Ergün, S., & Yigit, M.. Effects of dietary FARMARIN® XP supplement on immunological responses and disease resistance of rainbow trout (Oncorhynchus mykiss). Aquaculture, 2018, 496: 211-220.