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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*)

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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, and 0.075%CA+BS compared to the control and 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

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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, mycotoxin binders, photogenic or phytobiotic 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 [7-13]. 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 proteins and carbohydrates and metabolizes nutrients appropriately and produces B-group vitamins including B7 (biotin) and B12 (cobalamin) [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 (Aldrich 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) 10⁷ 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* 10⁷ cfu g⁻¹, cinnamic acid 0.025%+*B. subtilis* 10⁷ cfu g⁻¹, cinnamic acid 0.050%+*B. subtilis* 10⁷ cfu g⁻¹, cinnamic acid 0.075%+*B. subtilis* 10⁷ cfu g⁻¹ and cinnamic acid 0.150%+*B. subtilis* 10⁷ 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 [28]:

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 (H₂SO₄) 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:

Crude protein % = (discharge at titration - blind sample) × 0.1 (Normality of HCl solution) × 14.007 (Milliequivalent weight of nitrogen) × 6.25 (Factor) / sample weight × 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:

Crude fat amount % = weight change of glass bubble (g) / sample weight (g) × 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:

Crude ash content % = weight change of porcelain crucible (g) / sample weight (g) × 100

2.4 Biometric Indices

Biometric indices were calculated using the following equations:

Visceral fat index (VFI) = {wet weight of visceral fat (g) / [wet body weight (g) – wet weight of visceral fat (g)]} × 100

Hepatosomatic index (HSI) = {wet weight of liver (g) / [wet body weight (g) – wet weight of liver (g)]} × 100

Viscerosomatic index (VSI) = {wet weight of viscera and associated fat (g) / [wet body weight (g) – wet weight of viscera and associated fat (g)]} × 100

Bile-somatic index (BSI) = {wet weight of bile (g) / [wet body weight (g) – wet weight of bile (g)]} × 100

Spleen-somatic index (SSI) = {wet weight of spleen (g) / [wet body weight (g) – wet weight of spleen (g)]} × 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^[62].

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-

perimental diets compared to the control group ($p>0.05$).

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

	Experiment Groups					
	Control	Control+ <i>B.subtilis</i>	%0.025 CA+B. <i>subtilis</i>	%0.050 CA+B. <i>subtilis</i>	%0.075 CA+B. <i>subtilis</i>	%0.150 CA+B. <i>subtilis</i>
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	161.29±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)

	Experiment Groups					
	Control	Control+ <i>B.subtilis</i>	%0.025 CA+B. <i>subtilis</i>	%0.050 CA+B. <i>subtilis</i>	%0.075 CA+B. <i>subtilis</i>	%0.150 CA+B. <i>subtilis</i>
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±0.21	16.53±0.26	16.23±0.21	16.53±0.26	16.23±0.21
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

	Experiment Groups					
	Control	Control+ <i>B.subtilis</i>	%0.025 CA+B. <i>subtilis</i>	%0.050 CA+B. <i>subtilis</i>	%0.075 CA+B. <i>subtilis</i>	%0.150 CA+B. <i>subtilis</i>
*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
<i>B.subtilis</i>	-	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
<i>Enterobacteriaceae</i>	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					
	Control	Control+ <i>B.subtilis</i>	%0.025 CA+B. <i>subtilis</i>	%0.050 CA+B. <i>subtilis</i>	%0.075 CA+B. <i>subtilis</i>	%0.150 CA+B. <i>subtilis</i>
Feed pH	5.90	5.89	5.86	5.83	5.75	5.74
Stomach pH	6.81±0.04	6.88±0.03	6.92±0.02	6.87±0.04	6.91±0.02	6.93±0.03
Intestine pH	7.07±0.01	7.07±0.01	7.06±0.02	7.05±0.02	7.04±0.01	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					
	Control	Control+ <i>B.subtilis</i>	%0.025 CA+B. <i>subtilis</i>	%0.050 CA+B. <i>subtilis</i>	%0.075 CA+B. <i>subtilis</i>	%0.150 CA+B. <i>subtilis</i>
Trypsin (U/mg protein/ min)	1.66±0.26	1.57±0.16	1.66±0.15	1.43±0.14	2.02±0.19	1.53±0.12
Amylase (mU/mg pro- tein)	57.48±6.71 ^b	249.79±33.60 ^a	207.65±21.67 ^a	250.62±26.65 ^a	190.86±20.28 ^a	62.83±6.65 ^b
Lipase (uMol/mg pro- tein/min)	0.28±0.02	0.22±0.02	0.22±0.01	0.20±0.02	0.26±0.05	0.20±0.02
Alkaline phosphatase (U/mg protein/min)	0.23±0.03	0.21±0.03	0.26±0.03	0.17±0.02	0.20±0.03	0.24±0.03
Pepsin (U/mg protein/ min)	34.59±6.34	30.12±4.82	31.20±3.08	39.46±4.31	32.29±3.65	26.74±2.24

Table 6. Changes in internal organ indexes

Parameters	Experiment Groups					
	Control	Control+ <i>B.subtilis</i>	%0.025 CA+B. <i>subtilis</i>	%0.050 CA+B. <i>subtilis</i>	%0.075 CA+B. <i>subtilis</i>	%0.150 CA+B. <i>subtilis</i>
VSI	15.31±0.63 ^{ab}	16.21±0.59 ^{ab}	14.35±0.50 ^b	17.07±0.23 ^a	14.62±0.54 ^{ab}	15.68±0.86 ^{ab}
HSI	1.56±0.08 ^a	1.42±0.05 ^{ab}	1.34±0.06 ^{ab}	1.59±0.08 ^a	1.25±0.05 ^b	1.46±0.06 ^{ab}
VFSI	4.00±0.30 ^a	4.31±0.20 ^a	3.86±0.23 ^a	4.88±0.10 ^a	4.50±0.31 ^a	4.12±0.29 ^a
BSI	0.21±0.02 ^a	0.18±0.03 ^a	0.13±0.01 ^a	0.20±0.03 ^a	0.14±0.01 ^a	0.12±0.01 ^a
SSI	0.34±0.02 ^b	0.34±0.01 ^b	0.33±0.03 ^b	0.36±0.04 ^b	0.33±0.01 ^b	0.47±0.02 ^a
HSI	0.23±0.01 ^a	0.21±0.01 ^{ab}	0.19±0.02 ^{ab}	0.22±0.02 ^{ab}	0.17±0.02 ^b	0.22±0.01 ^{ab}

ment with a recent study on rainbow trout (*Oncorhynchus 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, Hassaann et al.^[6], 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*)^[1], 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*^[48]. 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 di-

gestive capacity and growth performance^[44,49,50]. 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 microflora^[54] of human or animal may cause food spoilage^[55]. Faecal coliforms 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 0.150CA + *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], 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 *Enterobacte-*

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.

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