Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (Oncorhynchus mykiss) reared at normal and high temperature

Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (*Oncorhynchus mykiss*)

Nicole Francesca Pelusio[1], Barbara Rossi[1], Luca Parma[1]*, Enrico Volpe[1], Sara Ciulli[1], Andrea Piva[1], Federica D'Amico[2], Daniel Scicchitano[2], Marco Candela[2], Pier Paolo Gatta[1], Alessio Bonaldo[1], Ester Grilli[1]

[1]Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, Bologna, Italy

[2]Unit of Microbial Ecology of Health, Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

*Corresponding author: luca.parma@unibo.it

Abstract

Organic acids (OA) and nature-identical compounds (NIC) such as monoterpenes and aldehydes are well-known growth and health promoters in terrestrial livestock while their application for fish production is recent and their mechanisms of action require further study. In addition, the encapsulation of these compounds represents a valuable strategy to increase stability and range, and makes it possible to target the intestinal tract where different beneficial effects can be exerted. Hence, this study tested the increasing dietary level (D0, D250, D500, D1000; 0, 250, 500 and 1000 mg kg feed^{-1} respectively) of a microencapsulated blend containing citric and sorbic acid, thymol and vanillin over 82

days on rainbow trout to assess the effects on growth, feed utilization, intestine cytokine gene expression and gut microbiota (GM). Furthermore, the effects on intestinal cytokine gene expression and GM were also explored after one week of exposure under suboptimal water temperature (23°C). OA and NIC improved specific growth rate (SGR) and feed conversion rate (FCR) during the second half (day 40-82) of the feeding trial, while at the end of the trial protein (PER) and lipid efficiency (LER) increased with increasing dietary level. GM diversity and composition and cytokine gene expression analysis showed no significant differences in fish fed with increasing doses of OA and NIC (82 days) demonstrating the absence of inflammatory activity in the intestinal mucosa. Although there were no statistical differences, GM structure showed a tendency in clustering D0 group separately from the other dietary groups and a trend towards reduction of *Streptococcus spp*. was observed in the D250 and D1000 groups. After exposure to suboptimal water temperature, lower GM diversity and increased gene expression of inflammatory intestinal cytokines were observed for both inclusions (D0 *vs*. D1000) compared to groups in standard condition. However, the gene up-regulation involved a limited number of cytokines showing the absence of a substantial inflammation process able to compromise the functional activity of the intestine. Despite further study should be conducted to fully clarify this mechanism, cytokines up-regulation seems correlated to the reduction of the GM diversity and, particularly, to the reduction of specific lactic acid bacteria such as *Leuconostoc*. The application of the microencapsulate blend tested can be a useful strategy to improve growth and feed utilization in rainbow trout under normal conditions, but further studies are needed to clarify its beneficial role under stressful conditions.

Keywords: rainbow trout, organic acid, nature-identical compounds, growth, feed utilization, cytokine gene expression, gut microbiome, suboptimal conditions.

1. Introduction

To secure optimal growth, gut health and function in farmed fish, there is now particular focus on various feed additives used for their nutritional value, and also for their health-promoting and disease-preventing properties. Functional feeds are typically supplied to ensure good health and to help the animal ward off pathogens during both normal and challenging farming conditions [1].

Organic acids (OA) are any organic carboxylic acids with the general structure R-COOH. These OA (and their salts) are manufactured by chemical synthesis or fermentation systems [2], and their utilization as feed additives is well known to improve performance and health in terrestrial livestock, especially in swine and poultry [3,4]. However, knowledge of their potential as growth promoters in aquaculture is more recent and less well-known due to limited research, but is expected to significantly increase in coming years [5]. Many advantages are reported in connection with lowered pH of feed and digesta owing to diet OA addition: (i) increase in feed hygiene by inhibiting growth of microbial acid-intolerant species, (ii) increase in digestive enzyme activity, leading to higher nutrient digestibility and feed utilization, (iii) modulate host gut microbiota and animal health [5,6]. Among the large variety of OA, dietary inclusion of citric acid was found to improve growth, feed intake, specific growth rate (SGR) and feed conversion ratio (FCR) of various aquaculture species such as red drum (*Sciaenops cellatus*), rainbow trout (*Oncohrynchus mykiss*), beluga sturgeon (*Huso huso*), yellowtail (*Seriola*

quinqueradiata), tilapia (*Oreochromis niloticus*), and red sea bream (*Pagrus major*) [7–14]. Concerning rainbow trout, while some studies on dietary citric acid displayed a positive effect on growth performance [9,12], a reduction in feed intake and no weight gain were found by other authors [15,16]. Another important OA is sorbic acid, a long chain unsaturated fatty acid known for its antimicrobial activity by inhibiting the microbial enzymatic apparatus and nutrient transport system [3]. Its growth-promoting effect was explored as combined in dietary blends within formic and benzoic acids and their respective salts, resulting in a significant increase in weight gain in rainbow trout [17].

During the past decade, botanicals and nature-identical compounds (NIC) have also gained great interest as novel animal feed additives for the positive effects reported on feed palatability and control of gut microbiota pathogens and for a possible direct effect on the immune system as recently reported in some fish species i.e. red hybrid tilapia (*Oreochromis niloticus* ♀ X *Oreochromis aureus* ♂) [18] , rainbow trout [19–21], tilapia [22] and zebrafish (*Danio rerio*) [18]. Among the most studied NIC, thymol is a monoterpene proposed as a growth promoter, antimicrobial and anti-inflammatory agent in mammals [23,24], whereas few studies have been conducted on fish species [18,25]. Similarly, vanillin, known as food and feed flavouring, also has potential antimicrobial activity by causing loss of membrane function and inhibiting cell respiration in several sensitive bacteria [26,27]. Using OA and NIC blends in aquafeeds could be an optimal strategy to test their potential synergistic effects on growth, nutrient utilization and gut health. In addition, the encapsulation process capable of protecting the compounds against interactions with the host, food ingredients, and environment may increase the reliability and predictability of their beneficial actions [5,28]. Moreover, to the best of our

knowledge the combination of citric and sorbic acids, vanillin and thymol has only been tested in sea bass (*Dicentrarchus labrax*) [29], while no data in rainbow trout are available.

For this reason, the aims of the present study of rainbow trout were: 1) to evaluate the effects of dietary increasing level of a blend of citric acid, sorbic acid, thymol and vanillin on growth, feed utilization, intestinal cytokine gene expression and gut bacterial community; 2) to explore the effects of this blend on intestinal cytokine gene expression and gut bacterial community after exposure to suboptimal water temperature conditions.

2. Materials and methods

2.1 Experimental diets

An extruded commercial diet (Ecofish 4, Veronesi SpA, Verona, Italy) (5 mm diameter pellet size - 42.1 ± 0.2 % crude protein, 20.1 ± 0.1 % crude lipid) was coated with increasing dose (D) levels (D0, D250, D500 and D1000; 0, 250, 500 and 1000 ppm respectively) of a blend of OA and NIC (providing 25% citric acid, 16.7% sorbic acid, 1.7% thymol and 1% vanillin) microencapsulated in a matrix of hydrogenated fats (Aviplus®-Vetagro SpA, Reggio Emilia, Italy; US patent # 7,258,880; EU patent # 1-391-155B1; CA patent # 2,433,484).

2.2 Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Rainbow trout specimens were obtained from an Italian fish farm (Pescicoltura Brenta snc, Vicenza, Italy). Animals were adapted to the facilities for 1 week before the start of the experiment. At the beginning of the trial, sixty fish (initial weight average 100.5 ± 0.4 g) were randomly distributed into each of twelve 800 L squared fibreglass tanks with a conical base. Each diet was randomly allocated and administered to triplicate groups over a period of 82 days. During the experiment, tanks were provided with tap freshwater and connected to a closed recirculation system (overall water volume: 15 m^3). The rearing system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25mJ/cm^2: 32m^3 h^{-1}, Blaufish, Barcelona, Spain), a biofilter (PTK 1200, Astralpool, Barcelona, Spain) and an active carbon filter. The water exchange rate within each tank was 100% every hour, while the overall water renewal amount in the system was 5% daily. During the trial, the temperature was kept at 15.0 ± 1.0 °C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant (10.0 ± 1.0 mg L^{-1}) by a liquid oxygen system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}) and nitrite (≤ 0.2 mg L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate ($NaOHCO_3$) was added on a daily basis to keep pH constant at 7.1–7.5 Feed was provided to satiation by oversupplying the feed via automatic feeders, twice a day (8:30, 16:30) for six days a week, while one meal was supplied on Sundays. Each meal lasted 1 hour, after which the uneaten pellets of each

tank were gathered, dried overnight at 105°C, and their weight was deducted for overall calculation.

2.3 Suboptimal rearing temperature

After the end of the feeding trial, fish belonging to D0 and D1000 groups were exposed to suboptimal rearing temperature over 7 days. The highest inclusion level (D1000) was chosen for comparison according to the results of growth and feed utilization achieved during the feeding trial. To this purpose water temperature was gradually increased (4-degree day^{-1}) up to 23°C and then maintained over one week. During this period, the feed and feeding procedures were provided as previously reported.

2.4 Sampling

At the beginning (day 0), in the middle (day 40) and at the end (day 82) of the feeding trial, all the fish were individually weighed. Before each sampling procedures, fish were anaesthetised (100 mg L^{-1}) or euthanized (300 mg L^{-1}) by MS222. Specific growth rate (SGR), feed intake (FI) and feed conversion rate (FCR) were calculated. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 15 fish and on a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency rate (PER), gross protein efficiency (GPE), lipid efficiency rate (LER) and gross lipid efficiency (GLE) were calculated. Furthermore, total body length, wet weight, viscera and liver weight were individually recorded for 5 fish per tank to determine condition factor (CF), viscerosomatic index (VSI) and hepatosomatic index (HSI) at the

end of the feeding trial. At the beginning (day 0, 15 fish in total), at the end of the feeding trial (day 82, 5 fish per $tank^{-1}$), and after the suboptimal rearing temperature period (5 fish per $tank^{-1}$), fish were sampled for pro-inflammatory and anti-inflammatory cytokine gene expression from the distal intestine. At the same time, 5 fish per tank were also sampled for gut bacterial community analysis.

All experimental procedures were evaluated by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.5 Analytical methods

Diets and whole body were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 °C overnight. Crude protein was determined as total nitrogen (N*6.25) after performing the Kjeldahl method. Total lipids were determined according to Bligh and Dyer's [30] extraction method. Ash content was estimated by incineration in a muffle oven at 450 °C overnight. Gross energy was determined by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, U.S.A).

2.6 Calculations

The formulae employed for growth performances were as follows: Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Feed conversion ratio (FCR) = feed intake / weight

gain. Viscerosomatic index (VSI) (%) = 100 * (viscera weight / body weight). Hepatosomatic index (HSI) (%) = 100 * (liver weight / body weight). Condition factor (CF) = 100*(FBW/length^3). Protein efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Lipid efficiency rate (LER) = (FBW – IBW) / lipid intake. Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish. Feed intake (FI) (% ABW^{-1} day^{-1})=((100 ∗ total ingestion)/(ABW))/days)) (where average body weight, ABW=(IBW+FBW)/2.

2.7 Cytokine gene expression analyses by real-time polymerase chain reaction

Total RNA was isolated from 50 mg of distal intestine samples stored in RNA Later (Sigma) using the NucleoSpin RNA extraction kit following the manufacturer's instructions. The RNA extraction protocol includes a treatment with DNAse I in order to remove genomic DNA. The first strand of cDNA was synthesized by reverse transcription using the GoScript® Reverse Transcriptase (Promega). cDNA concentration was quantified using a Qubit Fluorometer (ThermoFisher). Real-time PCR was performed with an ABI PRISM 7300 instrument (Applied Biosystems) using BRYT Green® GoTaq® qPCR (Promega). 10 ng of each cDNA sample was added to a reaction mix containing 2 x GoTaq® qPCR Master Mix (Promega), 300 nM of CXR and 200 nM of each primer. The primers used for β-actin (β-act), Interleukin 1β (IL-1β), 6 (IL-6), 8 (IL-8), 10 (IL-10), Tumor necrosis factor α (TNF-α), Transforming growth factor β (TGF-β), are shown in Table 1. Reaction mixtures were incubated for 2 min at 95 °C, followed by

50 cycles of 10 s at 95 °C, 30 s at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C (denaturation stage). Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. After these verifications, all cDNA samples were analyzed in triplicate. Negative controls with no template were always included in the reactions. For each sample, gene expression was normalized against beta-actin (β-actin) gene and expressed as $2^{-\Delta\Delta Ct}$, where ΔCt is determined by subtracting the β-actin Ct value from the target Ct. Gene expression of untreated and treated samples collected at time one (T1, day 82) and two (T2, day 89) were expressed as "fold changes" relative to untreated controls sampled at time zero (T0, day 0).

2.8 Gut bacterial community DNA extraction and sequencing

At the end of feeding trial, total bacterial DNA was extracted from pools of hindgut content obtained from 5 fish per tank (100 mg of hindgut content per fish) for a total of 12 (at day 82) and 6 (at day 89) samples, as previously reported in Parma [31]. Afterwards, the V3–V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 785R primers [32] with added Illumina adapter overhang sequences and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems) [33]. The PCR programme consisted of an initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, and 5 min at 72°C for the final elongation step [34]. PCR products were cleaned up for sequencing by using Agencourt AMPure XP magnetic beads as recommended in the Illumina protocol "16S Metagenomic Sequencing Library Preparation" for the MiSeq system. Afterwards,

Nextera Technology was used to construct indexed libraries by limited-cycle PCR reaction (Illumina, San Diego, CA). After a further clean up step as described above, libraries were normalized to 4 nM and pooled. The resulting pool was denatured with 0.2 N NaOH and diluted to a final concentration of 6 pM with a 20% PhiX control. Sequencing was carried out on Illumina MiSeq platform using a 2 × 250 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). After the sequencing process reads were processed using a pipeline combining QIIME2 [35] and PANDAseq [36]. Then, using DADA2 [37] and VSEARCH [38], reads were cleaned and clustered into OTUs at a 0.99 similarity threshold. Assignment was carried out by using the RDP classifier against Greengenes database (May 2013 release). During the bioinformatic analysis, one of the samples of D500 diet was excluded from the following analysis because of the low number of high quality sequences obtained. Alpha-diversity analysis were performed using OTU species count (observed_otus), Chao1 index for microbial richness and Shannon index for biodiversity. Beta-diversity was estimated by Bray-Curtis distances, which were used as input for principal coordinates analysis (PCoA).

2.9 Statistical analysis

All data are represented as mean ± standard deviation (SD). A tank was used as the experimental unit for analysing growth performance, and a pool of five fish was considered as the experimental unit for analysing carcass composition, whereas fifteen individual fish per treatment were used for analysing immunity response expression. Results on growth, nutritional indices and cytokine expression at the end of the feeding

trial (day 82) were analysed by applying linear regression model in order to measure the effect of the increasing doses of dietary OA and NIC on considered data, with a significance attributed for $p \leq 0.05$. Cytokine expression data in fish treated with D0 and D1000 on day 82 and 89 were analysed by two-way analysis of variance (ANOVA) considering diet and time as independent factors, and in case of significance ($p \leq 0.05$) Tukey's post hoc test was performed. Gut microbial statistical analysis of gut bacterial community was carried out by using R packages Stats and Vegan (https://www.r-project.org). In particular, the Kruskall-Wallis test was used to compare Alpha-Diversity among groups, whereas data separation in the PCoA was tested using a permutation test with pseudo F-ratios (function Adonis in the Vegan package). In addition, to compare the microbiota structure among different groups, at phylum and genus level, the Kruskal-Wallis test was used, and a paired Wilcoxon rank-sum test between groups when appropriate. P-values were adjusted using the false discovery rate (FDR), and a p-value ≤ 0.05 was considered as statistically significant. The rest of the statistical analyses were performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, USA).

3. Results

3.1 Growth and proximate composition

Growth performances are reported in Table 2. No significant dose effect was found concerning FBW, weight gain, FCR, SGR, FI and mortality during the first period of the trial (days 0-40). Regarding the second period (days 40-82), SGR ($p = 0.0294$) increased

at increasing OA and NIC level while a decreasing effect was observed on FCR ($p = 0.0439$). Results on biometric indices, nutritional indices and proximate whole-body composition are summarised in Table 3. No significant dose effects were found on VSI, HSI, CF and whole proximate composition. Concerning nutritional indices, PER ($p = 0.0202$) and LER ($p = 0.0156$) increased significantly at increasing dietary OA-NIC dose level. No significant dose effects were found in GPE and GLE.

3.2 Immune and inflammatory gene expression in intestinal mucosa

The gene expression of six genes involved in the immune and inflammatory response are presented in Figures 1 and 2. The comparison of gene expression in the intestine of fish fed different diets and sampled at the end of the feeding trial (day 82, time 1, T1) showed slight variations of some target genes analysed (Figure 1), but no significant dose effect was observed. The comparison of gene expression between D0 and D1000 groups before (T1) and after (day 89, time 2, T2) the exposure to suboptimal water temperature showed an upregulation of some pro-inflammatory genes analyzed (Figure 2). Particularly, IL-8 was significantly upregulated ($p < 0.001$) after the exposure to a suboptimal water temperature in both D0 and D1000 groups, while TNF-α was significantly upregulated in D1000 group ($p < 0.05$).

3.3 Gut bacterial community profiles

Twelve pools (at day 82) and six pools (at day 89) of the content of fish distal intestine were analysed to determine the gut microbial community of fish fed with increasing

inclusion levels of the dietary blend (OA + NIC). For each sample, the GM (gut microbiota) composition was defined by the next-generation sequencing of the V3-V4 hypervariable region of the 16S rRNA microbial gene. In order to assess whether the different diets D0, D250, D500 and D1000 can influence fish gut bacterial community at day 82, the beta-diversity of the GM ecosystem was evaluated by performing a Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distances among the gut bacterial community profiles (Fig. 3A). Even though no significant differences among dietary groups were detected (permutation test with pseudo-F ratios (Adonis); $p > 0.05$), PCoA showed a tendency of sample separation based on different diets, the D0 group clustered separately from the other dietary groups (i.e. D250, D500, D1000). More specifically, it was possible to identify a tendency of separation between D0 group and D500 group (pairwise Adonis permutation test; $p < 0.1$). In addition, compared to the other groups, D250 group showed better clustering, indicating a greater uniformity of this group compared to the others. Different metrics were used to estimate α-diversity of each sample, including OTU species count (observed_species), Chao1 index for microbial richness and Shannon index for biodiversity (Fig. 3B). However, no significant differences in the gut bacterial community α-diversity, according to the different diets, were detected (Kruskal-Wallis test; $p > 0.05$). In order to analyse further the GM composition of fish fed diets with different OA+NIC inclusions (i.e. D0, D250, D500, D1000), phylogenetic characterisation was highlighted in Fig. 3C-D. The GM of each group showed a similar profile in terms of phylum and genus taxonomic levels. In particular, at phylum level, the most abundant taxa observed were *Firmicutes*, *Proteobacteria* and *Actinobacteria*, which represented about 98% of the whole intestinal bacterial ecosystem (Fig. 3C). On the other hand, the genera most represented, all

belonging to *Firmicutes* phylum, were *Lactobacillus* (mean relative abundance ± SD; D0: 29.0% ± 2.5%; D250: 29.3% ± 1.6%; D500: 27.7% ± 0.4%; D1000: 31.3% ± 2.2%), *Leuconostoc* (D0: 18.2% ± 4.8%; D250: 20.1% ± 1.4%; D500: 19.7% ± 4.7%; D1000: 18.7% ± 4.7%) and *Streptococcus* (D0: 16.5% ± 0.7%; D250: 13.6% ± 0.9%; D500: 13.8% ± 0.1%; D1000: 13.6% ± 0.7%) (Fig. 3D). However, no significant differences (Wilcoxon rank-sum test; $p > 0.05$) among groups at phylum and genus level were detected between different diets (Supplementary Figure 1 A). Despite these primary results, a paired statistical analysis against each dietary group revealed several tendencies at the genus taxonomic level. Both *Vagococcus* and *Peptoniphilus* genera appeared to be most abundant in D250 group compared to D0 group (Wilcoxon rank-sum test; $p < 0.1$). On the other hand, for the genera *Streptococcus* and *Faecalibacterium* a reduction trend was observed in the D250 and D1000 groups compared to D0 group (Wilcoxon rank-sum test; $p < 0.1$). In addition, the *Clostridium* genus also seemed to be less abundant in the D250 compared to D0 group (Wilcoxon rank-sum test; $p < 0.1$) (Supplementary Figure 1).

Subsequently, in order to understand whether the exposure to a suboptimal water temperature can influence the gut bacterial ecosystem of fish treated with diet D0 and D1000 at both day 82 (T1) and 89 (T2) time-points, beta diversity analysis based on the Bray-Curtis distances among the gut bacterial community profiles of D0 (T2), D1000 (T2), D0 (T1) and D1000 (T1) groups was performed (Fig. 4A). In this case, the PCoA showed a significant separation between different groups in the two-dimensional space (permutation test with pseudo-F ratios (Adonis); $p = 0.003$), principally driven by the separation between D0 (T2) vs D0 (T1) (pairwise Adonis permutation test; $p = 0.001$), D0 (T2) vs D1000 (T1) ($p = 0.01$), D1000 (T2) vs D0 (T1) ($p = 0.001$), D1000(T2) vs

D1000 (T1) ($p = 0.007$) and D0 (T1) vs D1000 (T1) ($p = 0.01$). Concerning the α-diversity of each sample, different metrics were used for evaluation, including OTU species count (observed_otus), Chao 1 index and Shannon index (Fig. 4B). It was possible to observe a significant difference among groups before and after the exposure to a suboptimal water temperature (Kruskall-Wallis test; $p < 0.05$). All measures showed significantly less GM diversity under suboptimal conditions (T2) compared to non-suboptimal conditions (T1), for both types of diets (Kruskall-Wallis test; $p < 0.05$). The overall composition of fish gut bacterial community from D0 and D1000 groups before (day 82, T1) and after (day 89, T2) the exposure to suboptimal water temperature is represented in Fig. 4C-D. In particular, *Firmicutes*, *Proteobacteria* and *Actinobacteria* phyla dominated the intestinal bacterial community in all conditions and represented about 97% of the whole sample set (Fig. 4C). At the genus level, the most represented taxa all belonging to *Firmicutes* phylum, were *Lactobacillus*, *Leuconostoc*, *Streptococcus and Lactococcus*, below indicated as mean relative abundance ± SD. *Lactobacillus*: D0 (T2), 30.9% ± 0.6%; D1000 (T2), 29.0% ± 0.4%; D0 (T1), 29.0% ± 2.5%; D1000 (T1), 31.3% ± 2.2%). *Leuconostoc*: (D0 (T2), 7.4% ± 1.1%; D1000 (T2), 6.4% ± 1.2%; D0 (T1), 18.2% ± 4.8%; D1000 (T1), 18.7% ± 4.7%. Streptococcus: D0 (T2), 9.6% ± 2.5%; D1000 (T2), 9.7% ± 2.8%; D0 (T1), 16.5% ± 0.7%; D1000 (T1), 13.6% ± 0.7%. *Lactococcus*: D0 (T2), 5.2% ± 1.0%; D1000 (T2), 5.7% ± 0.4%; D0 (T1), 5.4% ± 0.9%; D1000 (T1), 6.1% ± 1.4% (Fig. 4D). A paired statistical analysis among groups did not highlight any significant difference at either phylum or genus level (Wilcoxon rank-sum test; $p > 0.05$) (Supplementary Figure 2).

However, at genus phylogenetic level it was possible to identify certain tendencies: both *Leuconostoc* and *Streptococcus* genera were less abundant in D0 (T2) and D1000 (T2)

groups, i.e. in a sub-optimal condition, compared to D0 (T1) and D1000 (T1) groups (Wilcoxon rank-sum test; $p < 0.1$). On the other hand, the *Erwinia* genus was found to be more abundant in the D0 (T2) group compared to D0 (T1) and D1000 (T1) groups (Wilcoxon rank-sum test $p < 0.1$) (Supplementary Figure 2).

4. Discussion

Feeding strategies addressing the use of organic acid and botanicals as potential growth and health promoters have attracted increasing interest in fish production [5,28]. In the present study, feeding rainbow trout with increasing dietary blend OA and NIC did not show a significant dose effect on growth performance in the first period of the trial. Similar outcomes were found in other studies conducted on salmonids species. Gao's study [39] did not report significant growth improvement of rainbow trout (233.32 g initial body weight) fed fishmeal or plant protein-based diets added with 10 g acid moiety/kg OA salt blends (mixture of sodium formate and butyrate, ratio 2:1) before and after feed extrusion. Another study by Bjerkeng [40] on OA salts blend (sodium acetate, sodium propionate and sodium butyrate, weight (w) concentration 5:5:2 w/w/w) at 0, 5 and 20 g Kg^{-1} dietary doses supplied to Atlantic salmon (*Salmo salar*) for 175 days displayed no significant effects on growth or apparent digestibility of macronutrients.

In the present work, even though there was no significant evidence of growth changes in the first period of the feeding trial, a significant improvement in performance was found in the second half of the study (days 40-82): Dietary OA and NIC inclusion led to a significant improvement of SGR and FCR. These results are also reinforced by the significant increasing of protein and lipid utilization as stated by PER and LER at the end

of the trial. In agreement with the present findings, De Wet [17] found an improvement in growth when rainbow trout fingerlings (40 g initial body weight) were fed diets supplemented with 10 or 15 g kg^{-1} OA blends, compared to the non-supplemented treatment in a four-month trial. Pandey [41] reported consistent better growth and mineral utilization in rainbow trout fed for 12 weeks with low fishmeal-based (15%) diets supplemented with different OA at 1% (citric, lactic, fumaric, formic and acetic), especially for citric and fumaric acid. More recently, Villumsen [42] found that the dietary supplementation of organic acids and β-glucan showed improved FCR and LER in rainbow trout juveniles after 37 days feeding. The work mentioned suggested that improved performance and digestibility of OA supplementation may be explained by lowered pH resulting in a higher dissociation of mineral compounds, reduced rate of gastric emptying and formation of chelated mineral complexes that can be easily absorbed. In addition, an improvement in digestive enzyme activities was also reported when 0.6% humic acid sodium salt supplementation was provided [43]. In Tran-Ngoc [44], Nile tilapias were fed with 2 g Kg^{-1} potassium diformate (KDF), 2 g Kg^{-1} calcium butyrate (CAB) and 4 g Kg^{-1} KDF and CAB mixture ration 1:1 dietary level inclusion for five weeks in normoxic state (6 mg L^{-1}), followed by another period of exposure to hypoxic (3 mg L^{-1}) condition. Outputs found significant differences only in the last 5 weeks under challenging conditions, where singular OAs feed inclusions enhanced growth and nutrient digestibility by improving intestinal morphology. Interestingly, the same OA and NIC blend of the present study showed induced significant higher average daily gain during the second half of a two-week trial compared to a control diet also in pigs [45]. Another explanation of growth improvement in the second period could be related to a possible gut microbiome modulation after one month of adaptation to feed,

leading to eubiosis within the host. It has been reported that the gut-secretion stimulating effect of botanicals also allows the microbiota to modulate and improve digestion and absorption of nutrients. This stimulation may provide a greater array of amino acids for protein synthesis and thus increase body protein content [46]. Organic acid and botanicals are known to modulate gut microbial community in fish species as reviewed by Ng [5] and Sutili [28] with several beneficial properties including prebiotic-like effect, direct action towards pathogens, reduced pathogen motility and invasion, interference with quorum sensing communication processes operating in different signal components of the bacterial cells, reduction of biofilm formation, and inhibition of extracellular protease activity and expression. Gut microbiota of rainbow trout using NGS technique have been extensively studied in relation to vegetal or animal ingredients to replace FM [47–50], while no data concerning the application of the employed OA and NIC are available. Our finding displayed *Firmicutes, Proteobacteria*, and *Actinobacteria* as the most abundant phyla while *Lactobacillus, Leuconostoc* and *Streptococcus* were the genus most represented. These results are consistent with previous finding in rainbow trout, indicating that the inclusion of plant ingredients favoured the presence of *Firmicutes* over *Proteobateria*, which were more abundant in marine-derived diets [47,48]. In addition, different genera of lactic acid bacteria such as *Lactobacillus and Leuconostoc*, all belonging to *Firmicutes*, are generally considered beneficial microorganisms associated with a healthy intestinal epithelium in trout and other fish species [31,51,52]. Our finding showed only a moderate impact for the different OA+NIC inclusion levels on the gut bacterial composition of sea bream as shown by PCoA analysis. However, even though no significant differences among dietary groups were detected, PCoA showed a tendency to clustering in D0 group separately from the other dietary groups. Although there were

no statistically significant differences affecting the relative abundance of specific taxa, a reduction trend for *Streptococcus* spp. was observed in the D250 and D1000 groups. Up to date, several bacterial species within the genus *Streptococcus spp* have been reported as important pathogens of fish species including salmonids [53,54]. Previous *in vivo* studies have found that dietary essential oils/plant extracts such as rosemary (*Rosmarinus officinalis*), mangrove plant (*Excoecaria agallocha*), Aloe (*Aloe vera*), and Shirazi thyme (*Zataria multiflora*) are able to counteract *Streptococcus* pathogens in fish species via inhibition of bacterial growth, repressing of cytotoxin production, or by enhancing the non-specific immunity and disease resistance [55–57]. Interestingly, Soltani [58] showed that Shirazi thyme was able to reduce growth of *Streptococcus iniae* from a rainbow trout disease outbreak and had a stronger effect on down-regulating streptolysin S-related gene compared to rosemary. The authors attributed this effect as being due to the higher content of monoterpenoid phenols such as thymol from the essential oil of thyme.

Temperature plays a key role in determining microbial diversity globally [59,60], and therefore may directly alter gut microbiomes in animals, especially in those that are unable to thermoregulate. Although the effects of temperature-induced changes in the gut microbiota on host colonization resistance have not been established yet, recent studies suggest that disruption of animal gut microbiota by temperature may reduce the resistance of hosts to invasion and colonization by pathogenic microorganisms [60]. In the present study, a significant reduction in the gut microbial diversity was obtained in both dietary treatments after one week's exposure to suboptimal temperature conditions at 23 °C. A few studies investigated the temperature effects and diet–temperature interactions on the diversity of gut bacteria in rainbow trout using high-throughput sequencing. Among these, Huyben [61] evaluating the effects of dietary substitution of fishmeal (FM) with

live yeast, and increasing water temperature, reported a lower gut microbial diversity in trout reared at warm temperatures (18 °C), compared to those reared in cold conditions (11 °C), while no effect on diet was obtained. In contrast, in yellowtail kingfish *Seriola lalandi* (a warm water marine fish species) the temperature of 20 °C was associated with a decrease in the richness and relative abundance of microbiota compared to fish kept at 26 °C [62]. The decreasing of alpha-diversity indices in fish species may lead to reduced competition for opportunistic or invading pathogens which may enter the gastrointestinal tract of fish via feed or water [52,63]. Reducing diversity GM in fish has been associated with stressful conditions such as rearing density [52] and feeding competition [64] and also characterized the GM of unhealthy rainbow trout experiencing bacterial kidney disease (BKD) [65].

The intestine has an important immunological role and constitutes a physical barrier against pathogens [66]. The gastrointestinal mucosal surface is a natural interface where the intestinal microbiota and antigens cross-talk with the host fish [67]. The gut mucosa is rich in immune cells such as lymphocytes, plasma cells, eosinophilic granulocytes, and macrophages which can elicit local responses [68]. Regulatory systems involved in acquired and innate immune systems is brought about by direct cell-to-cell contact involving adhesion molecules and by the production of chemical messengers. Chief among these chemical messengers are proteins called cytokines, which can induce a broad range of activities via multiple target cell types and through their redundancy, indicated by the overlap in activities among different cytokines [69]. Cytokines released by activated phagocytes are key factors in the inflammation process, particularly IL-1β, an important pro-inflammatory cytokine, interferons, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and several chemokines. When an inflammatory

response is induced, the cascade of cytokine secretion begins with the release of TNF-α; this stimulates the release of IL-1β, which is then followed by the release of IL-6. The initiation of inflammation leads to the release of a myriad of other cytokines, which include chemoattractants that signal neutrophils and macrophages to migrate to the site of infection (e.g. chemokines) [70]. In our study, during the feed trial (82 days) no significant differences were recorded in animals feed with increasing dose of OA and NIC. This finding proves that OA and NIC do not have inflammatory activities, pointing out the lack of the cytokine secretion cascade activation characteristic of the inflammation process. Accordingly, the growth performance and the gut microbiota composition recorded during the feeding trial did not show differences among tested diets. Thus, the establishment of a healthy microbiota plays an important role in the generation of immunophysiologic regulation in the host by providing crucial signals for the development and maintenance of the immune system [71]. The groups exposed to suboptimal water temperature showed no significantly different expression levels among the immune genes studied, except for IL-8 and TNF-α. Particularly, IL-8 is a potent inflammatory cytokine, which is known as neutrophil chemotactic factor and is produced by various immunocytes during oxidative stress and infections [72]. In addition, TNF-α is another inflammatory multifunctional cytokine synthesized by various kinds of cells and involved in the proliferation of immunocytes and in their migration, apoptosis, and phagocytic activity as well as in the expression of other pro-inflammatory cytokines [73]. The upregulation of IL-8 in D0 and D1000 groups and of the TNF-α in D1000 suggests the activation of a stress-condition triggered by the suboptimal temperature. In D0 and D1000 groups slight modifications in gut microbiota after the suboptimal temperature condition were also observed and these changes may have led to the upregulation of these

cytokines. In this context, the concomitant reduction of the LAB *Leuconostoc* should be mentioned. LAB are known to be involved in the modulation of topical and systemic immune systems being able to suppress the production of pro-inflammatory cytokines [74,75]. Contrary to our findings, rainbow trout exposed to stress conditions such as high stocking densities for 30 days showed different expression levels of pro-inflammatory cytokines. Particularly, the fish reared at high stocking densities (40 and 80 kg m^{-3}), showed a significant density-dependent down-regulation in the expression of IL-1β and IL-8 and the lowest level was observed in the highest stocking density group [76]. The differences in the results between the present study with the findings of Yarahmadi [76] are supported by the fact that short-term stress (acute) and long-term stress (chronic) have different effects on fish immune system [77]. However, in agreement with our findings, Castillo [78] reported that adrenaline at a concentration of 1 mM caused increased expression of TNF-α gene but suppressed the expression of IL-1β in gilthead sea bream (*Sparus aurata*) head kidney cells. Moreover, Caipang [79] reported that short-term overcrowding up-regulated several immune-related genes such as IL-1β, IL-8 and g-type lysozyme in the blood of Atlantic cod (*Gadus morhua*). In the present study temperature seems to exert a somewhat effect on gut health status as supported by both reduced diversity of GM and increased intestinal inflammatory cytokine gene expression. However, the up-regulation of IL-8 and TNF-α, and the absence of regulation of other pro- or anti-inflammatory genes suggest the lack of a substantial inflammation process able to compromise the functional activity of the intestine. The cytokine gene expression pattern obtained in this study may have been affected by the time course and the persistence of the stressor as previously suggested [80]. Furthermore, available literature suggests that feed additives may regulate inflammatory effects in an inconsistent pattern,

possibly depending on the differences of composition, dosage, quality, route, and exposure time [81].

5. Conclusions

In conclusion, the dietary microencapsulated blend of OA and NIC employed at the tested inclusions improved growth and feed utilization of rainbow trout. Significant dose effects on the improvement of SGR and FCR were evident during the second half of the trial (days 40-82), probably indicating that the duration of feed administration plays a role in inducing an improvement of digestive conditions or microbiome modulation. Gut microbiota (GM) diversity and composition and cytokine gene expression analysis showed no significant differences in fish fed with increasing doses of OA and NIC after 82 days, pointing out the lack of an inflammatory activity in the intestinal mucosa of rainbow trout fed increasing dietary blend. Although there was no statistical significance, microbiota analysis showed a tendency to clustering D0 group separately from the other dietary groups, and a reduction trend for *Streptococcus spp*. was observed in the D250 and D1000 groups. After exposure to sub-optimal conditions, a lower GM diversity and an increased intestinal inflammatory cytokine gene expression were observed for both types of diets (D0 vs. D1000) compared to groups in a standard condition. Although further studies should be conducted to fully clarify this mechanism, cytokine up-regulation seems correlated to the reduction of GM diversity and particularly to the reduction of specific bacterial genera such as *Leuconostoc*. The feeding of this microencapsulated blend of OA+NIC at the tested doses can be a useful strategy to

improve growth and feed utilization in rainbow trout. Further studies should be conducted to clarify their beneficial role in counteracting stressful conditions.

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Table 1. Primer sequences used for gene expression analyses.

Gene	Abbreviation	GenBank ID	Primer sequence (5'- 3')	Amplicon (bp)	References
β-actin	β-act	EZ908974	GCCGGCCGCGACCTCACAGACTAC CGGCCGTGGTGGTGAAGCTGTAAC	73	[82]
Interleukin 1 β	IL-1β	AJ223954	CTCTACCTGTCCTGCTCCAAA ATGTCCGTGCTGATGAACC	194	[83]
Interleukin 6	IL-6	DQ866150	CAATCAACCCTACTCCCCTCT CCTCCACTACCTCAGCAACC	91	[83]
Interleukin 8	IL-8	AJ279069	AGAATGTCAGCCAGCCTTGT TCTCAGACTCATCCCCTCAGT	69	[84]
Interleukin 10	IL-10	AB118099	CGACTTTAAATCTCCCATCGAC GCATTGGACGATCTCTTTCTTC	70	[84]
Tumor necrosis factor α	TNF-α	AJ277604 AJ401377	CCACACACTGGGCTCTTCTT GTCCGAATAGCGGGAAATAA	128	[85]
Transforming growth factor β	TGF-β	X99303	TCCGCTTCAAAATATCAGGG TGATGGCATTTTCATGGCTA	71	[82]

Table 2. Growth performance and feed intake of rainbow trout fed increasing dietary level of organic acids and nature-identical compounds

	Experimental diets					
	D0	D250	D500	D1000	R^2	*P*-value
Time range day 0 – 40						
IBW (g)	100.1 ± 3.5	100.8 ± 4.0	100.1 ± 3.1	100.9 ± 2.4		n.s.
FBW (g)	193.9 ± 5.0	200.6 ± 8.4	193.4 ± 12.6	193.6 ± 10.2		n.s.
Weight gain (g)	93.7 ± 7.9	99.8 ± 4.4	93.3 ± 12.1	92.7 ± 11.4		n.s.
SGR (% day^{-1})	1.65 ± 0.14	1.72 ± 0.01	1.64 ± 0.16	1.63 ± 0.17		n.s.
FCR	1.06 ± 0.07	1.06 ± 0.01	1.08 ± 0.05	1.09 ± 0.05		n.s.
FI	1.67 ± 0.03	1.73 ± 0.03	1.71 ± 0.07	1.69 ± 0.05		n.s.
Survival (%)	100.0 ± 0.0	100.0 ± 0.0	99.4 ± 1.0	99.4 ± 1.0		n.s.
Time range day 40 – 82						
IBW (g)	190.8 ± 3.4	199.2 ± 9.7	194.4 ± 11.4	193.7 ± 11.0		n.s
FBW (g)	296.9 ± 6.6	319.7 ± 8.0	309.8 ± 16.0	320.1 ± 13.7		n.s
Weight gain (g)	106.0 ± 7.7	120.5 ± 10.0	115.4 ± 5.0	126.4 ± 2.9		n.s
SGR (% day^{-1})	1.05 ± 0.07	1.13 ± 0.11	1.11 ± 0.03	1.20 ± 0.04	*0.331*	*0.029*
FCR	1.24 ± 0.09	1.15 ± 0.06	1.18 ± 0.01	1.12 ± 0.03	*0.281*	*0.043*
FI	1.27 ± 0.07	1.26 ± 0.05	1.27 ± 0.01	1.27 ± 0.02		n.s
Survival (%)	97.2 ± 0.96	99.4 ± 0.1	99.0 ± 1.0	98.3 ± 0.6		n.s

Data are given as the tanks mean (n=3) ± SD. n.s.: non-significant ($p > 0.05$).
D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D250 = 250 ppm; D500 = 500 ppm; D1000= 1000 ppm).
IBW = Initial body weight.
FBW = Final body weight.
FI = Feed intake (FI) (% ABW^{-1} day^{-1})=((100 ∗ total ingestion)/(ABW))/days)) (where average body weight, ABW=(IBW+FBW)/2;
FCR = Feed conversion rate = feed intake / weight gain.
SGR = Specific growth rate (% day-1) = 100 * (ln FBW- ln IBW) / days.

Table 3. Biometric indices, body composition and nutritional indices of rainbow trout fed increasing dietary level of organic acids and nature-identical compounds over 82 days

	Experimental diets					
	D0	D250	D500	D1000	R^2	*P*-value
Biometric indices						
VSI	13.23 ± 2.53	13.77 ± 1.74	13.11 ± 2.84	14.23 ± 1.75		n.s.
HSI	1.27 ± 0.28	1.42 ± 0.33	1.41 ± 0.28	1.41 ± 0.41		n.s.
CF	1.36 ± 0.48	1.28 ± 0.11	1.25 ± 0.12	1.24 ± 0.12		n.s.
Whole body composition, %						
Protein	16.48 ± 0.25	16.32 ± 0.23	15.94 ± 0.54	16.30 ± 0.24		n.s.
Lipid	15.74 ± 1.34	18.21 ± 0.54	17.70 ± 0.35	16.19 ± 0.85		n.s.
Ash	1.87 ± 0.27	2.10 ± 0.30	1.98 ± 0.30	1.94 ± 0.19		n.s.
Moisture	63.59 ± 1.98	60.69 ± 1.98	62.15 ± 0.35	64.44 ± 1.01		n.s.
Nutritional indices						
PER	2.05 ± 0.07	2.13 ± 0.04	2.11 ± 0.03	2.18 ± 0.07	*0.375*	*0.020*
LER	4.28 ± 0.14	4.45 ± 0.09	4.42 ± 0.07	4.56 ± 0.14	*0.404*	*0.015*
GPE	32.98 ± 0.66	33.92 ± 0.53	32.43 ± 2.14	34.68 ± 1.02		n.s.
GLE	81.21 ± 7.11	99.29 ± 5.25	95.94 ± 2.42	88.32 ± 6.19		n.s.

Data are given as the mean (n=15 for VSI, HIS, CF) ± SD. n.s.: not significant ($p > 0.05$).
D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D250 = 250 ppm; D500 = 500 ppm; D1000= 1000 ppm).
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
CF = Condition factor = 100*(FBW/length^3).
PER = Protein efficiency rate = ((FBW-IBW)/protein intake).
LER= Lipid efficiency rate = ((FBW-IBW)/lipid intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.
GLE = Gross lipid efficiency = 100*[(%final body lipid*FBW) - (%initial body lipid*IBW)]/total lipid intake fish.

Key to Figures

Figure 1. Immune and inflammatory cytokine gene expression in intestinal mucosa of rainbow trout fed increasing dietary blend (organic acids and natural identical compounds) levels over 82 days. Data are given as 15 individuals per diet. In each graph, significance is attributed to $P \leq 0.05$. D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D250 = 250 ppm; D500 = 500 ppm; D1000= 1000 ppm. IL-1β = Interleukin 1β; IL-6 = Interleukin 6; IL-8 = Interleukin 8; TNFα = Tumor necrosis factor α; TGFβ = Transforming growth factor β; IL-10 = Interleukin 10.

Figure 2. Immune and inflammatory cytokine gene expression in intestinal mucosa of rainbow trout fed dietary blend (organic acids and natural identical compounds) before (day 82, time 1, T1) and after (day 89, tme 2, T2) exposure to suboptimal water rearing temperature of 23°C for 7 days. Data are given as 15 individuals per diet. In each graph, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D1000= 1000 ppm. IL-1β = Interleukin 1β; IL-6 = Interleukin 6; IL-8 = Interleukin 8; TNFα = Tumor necrosis factor α; TGFβ = Transforming growth factor β; IL-10 = Interleukin 10.

Figure 3 Gut microbiota diversity and composition of rainbow trout fed for 82 days with increasing dietary blend levels (organic acids and natural identical compounds) (i.e. D0, D250, D500, D1000). Principal Coordinates Analysis (PCoA) based on Bray-Curtis

distances between samples taken from each diet groups is highlighted in panel A. No significant separation was observed among groups (permutation test with pseudo-F ratios (Adonis); $p > 0.05$). (B) Boxplots showing alpha diversity values for each dietary group, measured by OTU species count (observed_otus), Chao1 index and Shannon index. No significant differences were observed among groups for all the metrics (Kruskal-Wallis test; $p > 0.05$). Barplots showing the GM composition at phylum and genus taxonomic level for all the different dietary interventions are displayed in panel C and D respectively. Only phyla with a relative abundance ≥ 0.5% in at least 9% of samples, and genera with relative abundance ≥ 0.5% in at least 9% of samples are represented.

Figure 4 Gut microbiota diversity and composition of rainbow trout fed with D0 and D1000 diets before (day 82, T1) and after (day 89, T2) exposure to suboptimal water rearing temperature of 23°C for 7 days. Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances between samples taken from each group is displayed in panel A. A significant separation among groups was observed (permutation test with pseudo-F ratios (Adonis); $p = 0.003$). (B,) Boxplots showing alpha diversity values, measured by OTU species count (observed_otus), Chao1 index and Shannon index. All metrics showed lower GM diversity in groups exposed to a sub-optimal condition (T2) compared to groups in a standard condition (T1), for both type of diets (D0 vs. D1000) (Kruskal-Wallis test; $p < 0.05$). Barplots highlighting the GM composition at phylum-and genus level of different conditions are showed in panel C and D respectively. Only phyla with a relative abundance ≥ 0.5% in at least 8% of samples, and genera with relative abundance ≥ 0.5% in at least 8% of samples are represented.

Figure 1

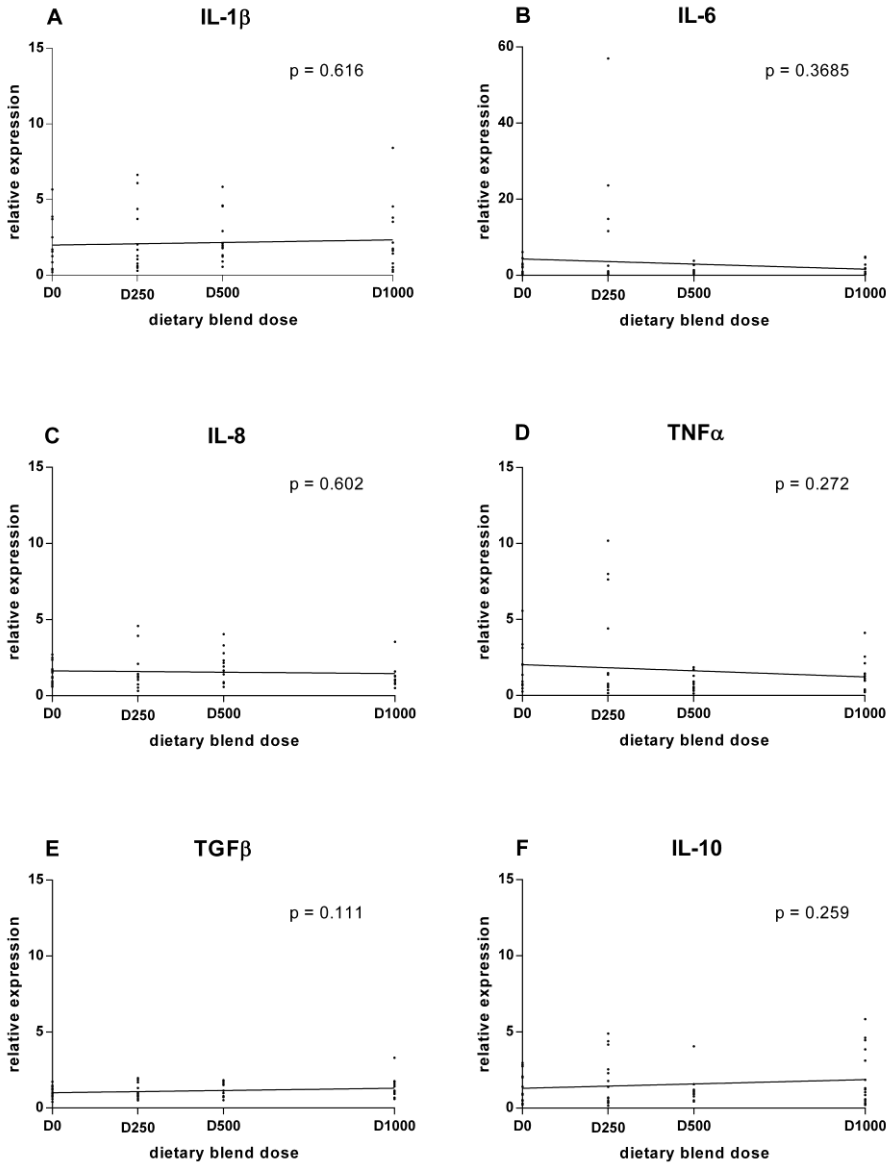

A
IL-1β
p = 0.616
relative expression
dietary blend dose
B
IL-6
p = 0.3685
C
IL-8
p = 0.602
D
TNFα
p = 0.272
E
TGFβ
p = 0.111
F
IL-10
p = 0.259
D0
D250
D500
D1000

Figure 2

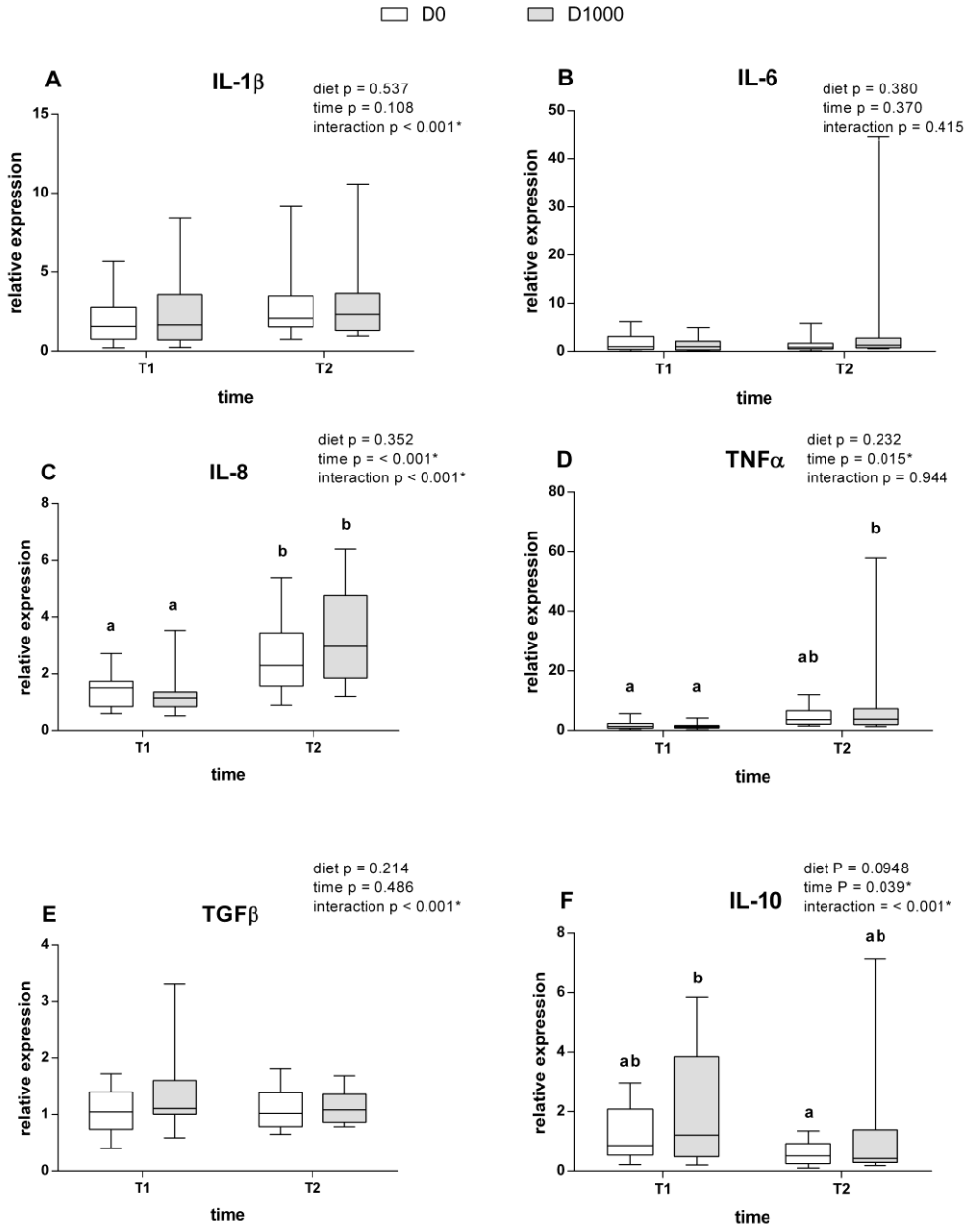

D0
D1000
A
IL-1β
diet p = 0.537
time p = 0.108
interaction p < 0.001*
relative expression
time
T1
T2
B
IL-6
diet p = 0.380
time p = 0.370
interaction p = 0.415
C
IL-8
diet p = 0.352
time p = < 0.001*
interaction p < 0.001*
D
TNFα
diet p = 0.232
time p = 0.015*
interaction p = 0.944
E
TGFβ
diet p = 0.214
time p = 0.486
interaction p < 0.001*
F
IL-10
diet P = 0.0948
time P = 0.039*
interaction = < 0.001*

Figure 3

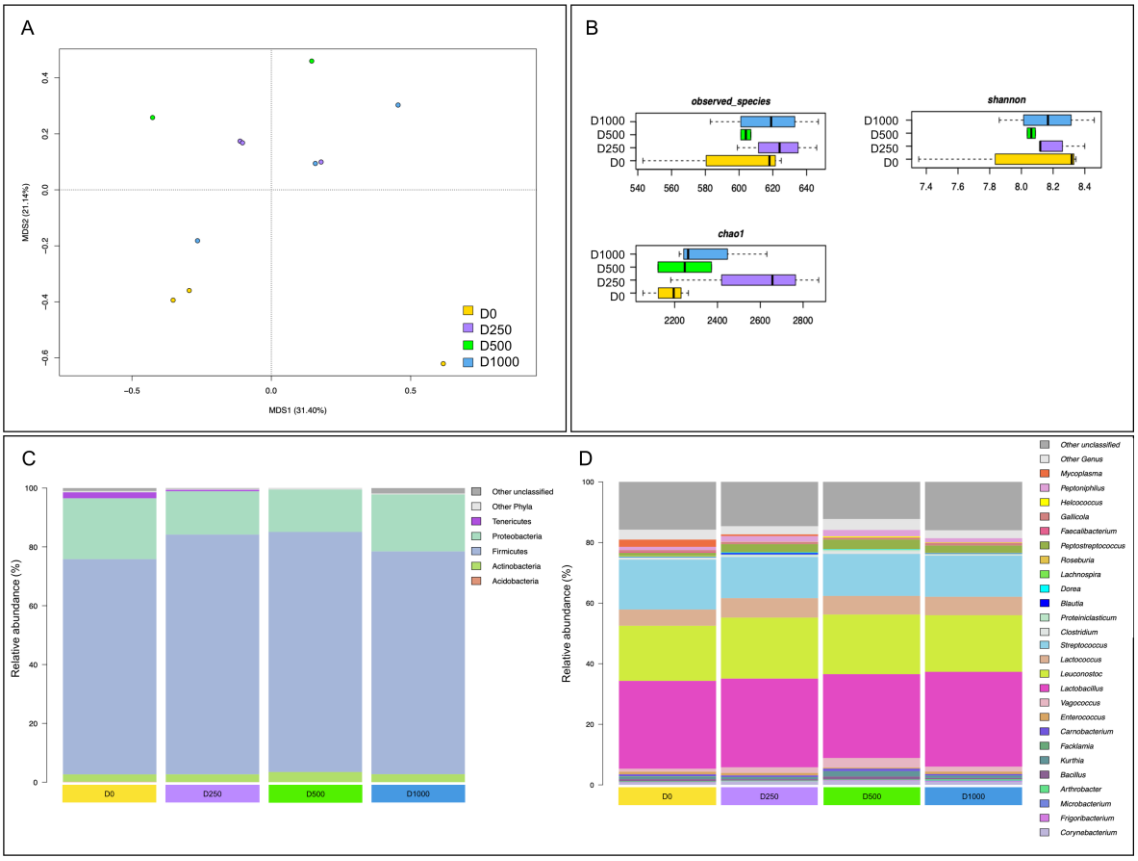
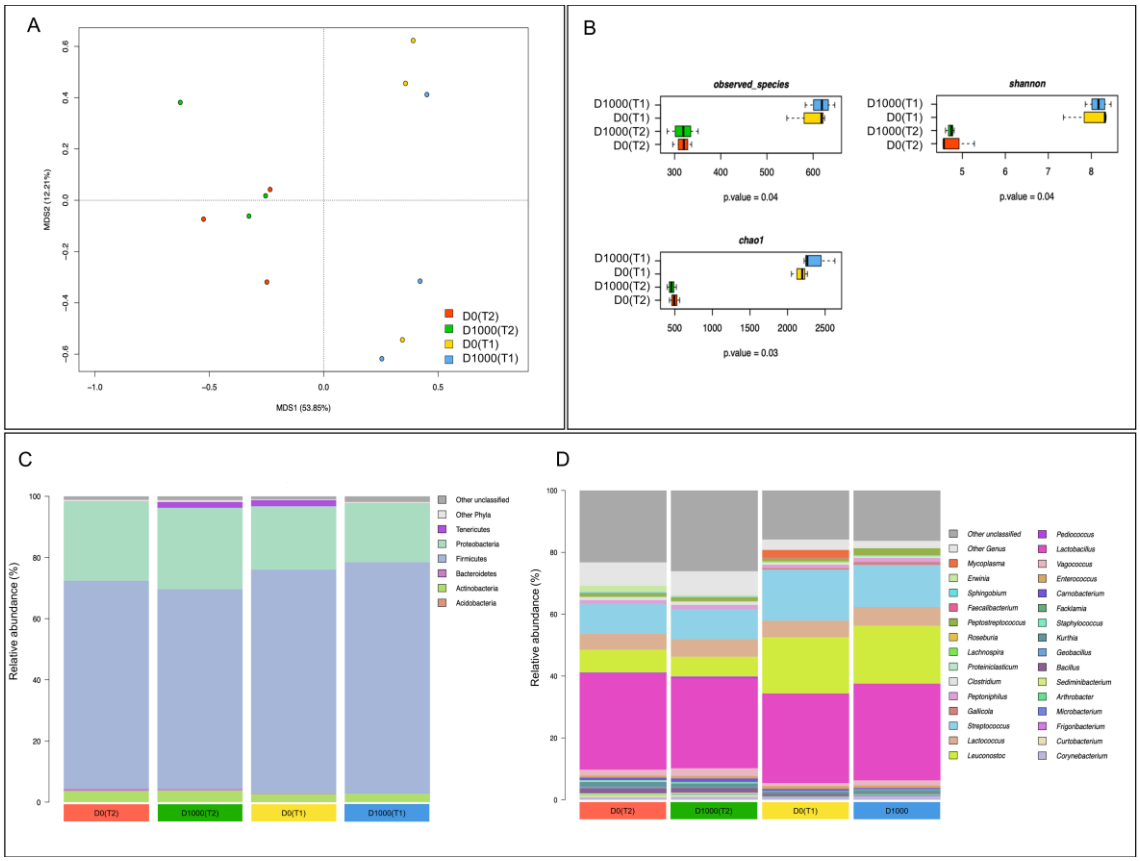
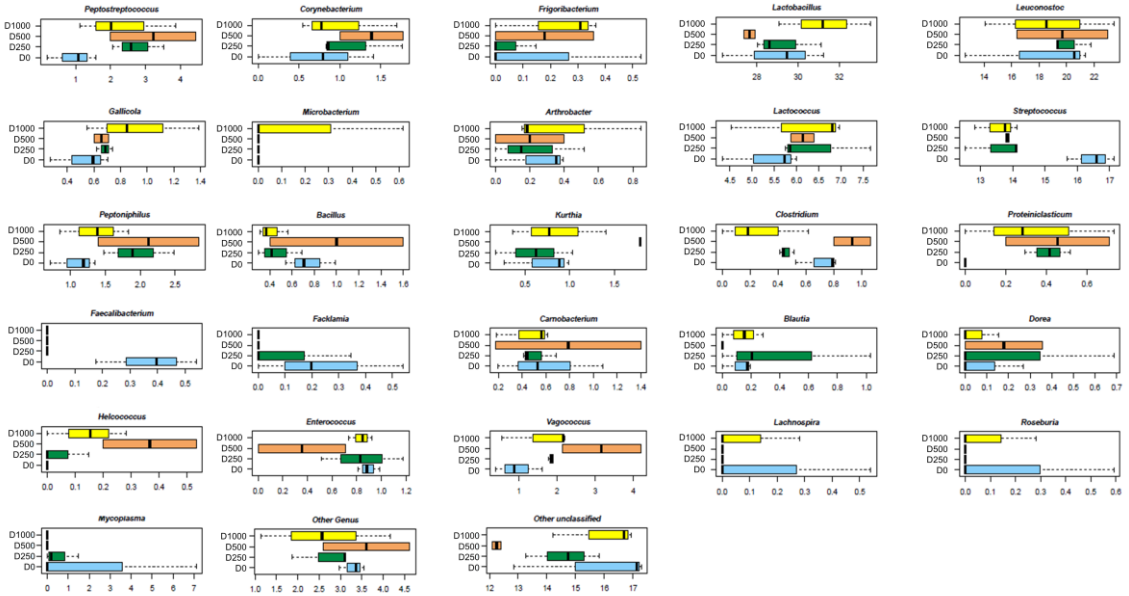

A
MDS2 (21.14%)
MDS1 (31.40%)
D0
D250
D500
D1000
B
observed_species
shannon
chao1
C
Relative abundance (%)
Other unclassified
Other Phyla
Tenericutes
Proteobacteria
Firmicutes
Actinobacteria
Acidobacteria
D
Relative abundance (%)
Other unclassified
Other Genus
Mycoplasma
Peptoniphilus
Helicococcus
Gallicola
Faecalibacterium
Peptostreptococcus
Roseburia
Lachnospira
Dorea
Blautia
Proteiniclasticum
Clostridium
Streptococcus
Lactococcus
Leuconostoc
Lactobacillus
Vagococcus
Enterococcus
Carnobacterium
Facklamia
Kurthia
Bacillus
Arthrobacter
Microbacterium
Frigoribacterium
Corynebacterium

Figure 4


A
MDS2 (12.21%)
MDS1 (53.85%)
D0(T2)
D1000(T2)
D0(T1)
D1000(T1)
B
observed_species
D1000(T1)
D0(T1)
D1000(T2)
D0(T2)
p.value = 0.04
shannon
p.value = 0.04
chao1
p.value = 0.03
C
Relative abundance (%)
Other unclassified
Other Phyla
Tenericutes
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria
Acidobacteria
D0(T2)
D1000(T2)
D0(T1)
D1000(T1)
D
Relative abundance (%)
Other unclassified
Other Genus
Mycoplasma
Erwinia
Sphingobium
Faecalibacterium
Peptostreptococcus
Roseburia
Lachnospira
Proteiniclasticum
Clostridium
Peptoniphilus
Gallicola
Streptococcus
Lactococcus
Leuconostoc
Pediococcus
Lactobacillus
Vagococcus
Enterococcus
Carnobacterium
Facklamia
Staphylococcus
Kurthia
Geobacillus
Bacillus
Sediminibacterium
Arthrobacter
Microbacterium
Frigoribacterium
Curtobacterium
Corynebacterium
D0(T2)
D1000(T2)
D0(T1)
D1000

Supplementary fig 1



Supplementary fig 2