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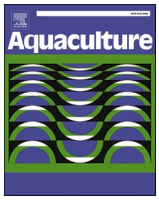

Interactions between nutritional programming, genotype, and gut microbiota in Atlantic salmon: Long-term effects on gut microbiota, fish growth and feed efficiency

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ABSTRACT

Nutritional programming (NP) is a tool for developing adaptive changes that can be expressed in adulthood by exposing individuals to a stimulus early in life. This study investigated the interactions between nutritional programming (NP), genotype and gut microbiota in Atlantic salmon (*Salmo salar*) across the life cycle, to potentially improve feed efficiency and fish health. Approximately 5100 eggs from six families characterised by high (HP) or low pigment retention (LP) were incubated and divided into four groups (HPM, HPV, LPM, LPV) that received a stimulus diet based on marine (M) (61 % fishmeal and 8 % fish oil) or vegetable (V) (5 % fishmeal, plant proteins and rapeseed oil) ingredients. This stimulus phase lasted three weeks, followed by a 49-week freshwater intermediate phase with fish fed a commercial feed subsequent to seawater transfer. In seawater, the fish were initially fed a commercial feed for 13 weeks and then switched to a plant-based "challenge" diet with approximately 3 % EPA + DHA until the end of the experiment, at 101 weeks, at which point fish were 4 kg. During the study, survival rates, SGR, and FCR were monitored. Samples for microbiota analysis were collected at T0 (after the stimulus), T1 (before the challenge), T2 (challenge, after the feed change), and T3 (end of the feeding trial). Gut and feed microbiota were analysed by bacterial DNA extraction, Illumina NGS library preparation and raw sequencing data analysis using QIIME 2 and PICRUSt software.

Gut microbiota composition changed with fish age, independent of NP and pigmentation genotype, emphasising the importance of developmental stage. Early diet influenced beta diversity and increased the number of specific bacteria, but these changes decreased with time. NP influenced the gut microbiota during the stimulus phase but not during the challenge phase, showing that the current diet has a greater influence than the earlier diet. Some microbial genera were associated with different genotypes and diets, suggesting interactions between genotype and stimulus diet. Differences in the metabolic potential of the gut microbiota due to the stimulus diet were observed but were not associated with differences in growth and feed utilisation.

The study concludes that early nutritional programming with a plant-based diet has a transient effect on growth and gut microbiota, with long-term growth performance being more strongly influenced by pigmentation genotype. Further studies on the interactions between genotype, diet and microbiota are required.

Abbreviations: BW, Body weight; DHA, Docosasaenoic acid; DPH, Days post-hatching; EPA, Eicosapentaenoic acid; FA, Fatty acid; FE, Feed efficiency; FI, Feed intake; FM, Fish meal; FO, Fish oil; FW, Freshwater; G, Genotype; HP, High pigment retention; LAB, Lactic acid bacteria; LC-PUFA, Long-chain polyunsaturated fatty acid; LP, Low pigment retention; M, Marine; PCoA, Principal coordinate analyses; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PPC, Pea protein concentrate; S, Stimulus; SGR, Specific growth rate; SNP, Simulated natural photoperiod; SPC, Soya protein concentrate; STAMP, Statistical Analysis of Metagenomic Profiles; SW, Sea water; V, Vegetable; WPH, Weeks post-hatching..

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1. Introduction

Global aquaculture production reached almost 123 million tonnes in 2020 (~7 million tonnes more than in 2018), and this growth is expected to continue (FAO, 2022). Among finfish, Atlantic salmon (*Salmo salar*) has emerged as the most important species farmed in marine aquaculture (FAO, 2022). This is partly because salmon flesh is considered an important source of omega-3 (*n*-3) fatty acids (FA) in humans, particularly the *n*-3 long-chain polyunsaturated FA (*n*-3 LC-PUFA): eicosapentaenoic acid (EPA; 20.5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which play an important role in human health (Sprague et al., 2016; Tocher, 2015). However, Atlantic salmon cannot biosynthesise these important LC-PUFAs in sufficient quantities, so they must be supplied in the diet (Tocher et al., 2019).

Feed is the most important economic and environmental factor in Atlantic salmon farming, since its manufacture involves various sustainability aspects including the use of high-quality globally traded ingredients (Aas et al., 2022). As a carnivorous fish, Atlantic salmon is traditionally supplied with the required n-3 LC-PUFA by adding fishmeal (FM) and fish oil (FO) to the feed (NRC, 2011). However, the conventional resources used to produce FM and FO for aquafeeds are in decline (Bell et al., 2010; FAO, 2022). Hence, diet composition has shifted significantly in recent years from the original raw marine materials, FM and FO, to ingredients of terrestrial origin (Aas et al., 2022; Ytrestøyl et al., 2015). Ingredient availability in addition to the changes in nutritional requirements throughout the fish life cycle, different farming systems and fluctuations in raw materials price, among other factors, make feed a challenging element of the production cycle. Thus, the assessment of new sustainable raw materials is crucial for the development of new high-quality feeds for Atlantic salmon. However, most of these novel ingredients are poor sources of EPA and DHA, or lack them altogether, prompting the industry to rely on inclusion of FO and/or FM to meet the requirements for these fatty acids (Betancor et al., 2017; Emery et al., 2016).

Nutritional programming (NP) could be a useful strategy to physiologically enhance the acceptance of new raw materials by fish (Geurden et al., 2013). This mechanism could further improve EPA and DHA retention and even boost *n*-3 LC-PUFA biosynthetic pathways in some fish species (Vagner et al., 2009). NP is a tool to initiate adaptive changes that can be expressed in adulthood by exposing individuals to a stimulus early in life (Lucas, 1998). Indeed, previous studies have shown that it is possible to programme Atlantic salmon with plant ingredients (Clarkson et al., 2017; Vera et al., 2017). The interactions between NP and genotype have been studied in rainbow trout (*Oncorhynchus mykiss*) (Geurden et al., 2013) and gilthead sea bream (*Sparus aurata*) (Naya-Català et al., 2023). However, in Atlantic salmon it is not yet clear whether the NP effects can also be maintained or reproduced in the seawater (SW) phase of production. In addition, there is a lack of studies in Atlantic salmon investigating the interaction of dietary programming with other factors, including genotype, and their potential effects on the gut microbiota.

Indeed, the relationship between NP and the gut microbiota of fish is an area of growing interest in understanding how early nutritional interventions can positively influence fish growth, health and development. Although the gut microbiota of fish has been studied in several species, including model, commercially farmed and wild fish, the relationship with NP is still poorly understood. What is known is that the gut microbiota plays an important role in fish health and disease and can be influenced by various factors, such as diet, water quality and genotype (Luan et al., 2023; Ringø et al., 2022). Indeed, our team has recently demonstrated a synergistic effect of diet and genotype on gut microbial communities in European seabass (*Dicentrarchus labrax*) selected for high growth and fed a novel diet without marine raw materials (Rimoldi et al., 2023; Torrecillas et al., 2023).

To date, a single study has investigated the effects of NP on Atlantic salmon gut microbiota, revealing sustained change in gut communities in later stages following a plant-rich "stimulus" diet, although this work only covered a short period during freshwater rearing (Tawfik et al., 2024). Other studies investigating the relationship between NP and gut microbiota modulation include those conducted in largemouth bass (*Micropterus salmoides*) (Kwasek et al., 2021), zebrafish (*Danio rerio*) (Kwasek et al., 2022; Patula et al., 2021) and ballan wrasse (*Labrus bergylta*) (Malzahn et al., 2022). Two of these investigated the effect of NP on the utilisation of plant proteins, growth and gut microbiota of fish in the preadult or adult stage (Kwasek et al., 2021; Patula et al., 2021). An unconventional NP strategy was also tested in zebrafish by feeding soybean meal to the parents to improve the utilisation of soybean meal in the offspring (Kwasek et al., 2022). However, in all these studies, the gut microbiota did not appear to be part of the NP mechanism, as no change in gut microbiota was associated with NP. Similarly, rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) showed no sustained changes in their gut microbiota when fed different amounts of plant proteins at first feeding (Michl et al., 2019; Michl et al., 2017).

Therefore, to further investigate possible relationships between NP and the gut microbiota in other species, the aim of the present study was to characterise the response of the gut microbiota to NP and the interactions with genotype in Atlantic salmon.

2. Materials and methods

2.1. Ethical statement

All experimental procedures were carried out in accordance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practise, January 1997) under project licence PBBB474D5 in accordance with EU regulation (EC Directive 86/609/EEC). All experiments were subject to ethical review by the Animal Welfare and Ethical Review Board (AWERB) of the University of Stirling (AWERB/1920/093).

2.2. Experimental diets

All feeds used during the stimulus and challenge phase were formulated and manufactured by Skretting ARC, Norway. Two different isolipidic, iso-nitrogenous and isoenergetic feeds were used in the stimulus phase. A marine diet (M), which served as a control, contained 61 % FM as the main protein source and 8 % FO as the lipid source. In contrast, the plant-based diet (V) contained a lower amount of FM (5 %) and a mixture of plant proteins, including wheat gluten, soya protein concentrate (SPC) and pea protein concentrate (PPC), to name a few. In addition, the V diet contained rapeseed oil as the main source of lipids (0 % FO). The proximate composition and EPA and DHA content of the stimulus and challenge diets are shown in Table 1. The challenge diets were all plant-based and had a very similar composition to the stimulus V diet (5 % FM and 0 % FO). However, they also contained a mixture of microalgae, linseed, palm and camelina oils as the predominant lipid source.

Table 1
Fish diets.

Experimental phase	Stimulus phase		Seawater phase			
Diets	M	V	A	B	C	D
Size (mm)	0.5	0.5	4.0	7.0	9.0	9
Analysed proximate composition						
Lipid – crude (%)	15.1	15.2	27.7	30.0	34.0	37.8
Protein – crude (%)	52.4	54.0	44.0	42.1	37.3	34.0
Energy – gross (Mj/kg)	21.0	21.9	22.7	23.9	23.1	25.5
All fatty acids (% total fatty acids)						
EPA (20:5n-3)	8.8	0.8	1.1	1.1	0.9	0.8
DHA (22:6n-3)	12.6	1.1	2.0	2.1	2.1	2.2
EPA + DHA	21.4	1.9	3.1	3.2	3.1	3.0

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

2.3. Fish stock and culture conditions

Approximately 5100 eggs from six families (Mowi Breeding Programme, Ireland) were delivered to to the Niall Bromage Freshwater Research Unit (NBFRU) of the Institute of Aquaculture in the last week of January 2020 and kept in a flow-through system in freshwater (FW). Three of the families were characterised by high pigment retention (HP; estimated breeding value, EBV > 115) and three by low pigment retention (LP; EBV < 85) genotypes. Half of the eggs were incubated at 3.0 ± 0.6 °C, while the other half were kept at 5.7 ± 0.7 °C for three weeks until the two groups reached ~490°d. At this point, both genotypes were equally divided into 4 trays, 2 for HP and 2 for LP, and each was assigned either a M or V stimulus diet. After hatching and before first feeding took place, the animals were transferred into four flow through tanks with a volume of 400 l each.

2.4. Feeding trial

2.4.1. Freshwater (FW)

The stimulus phase comprised the first three weeks of exogenous feeding in FW (Fig. 1) and was carried out at the NBFRU. As described above, each genotype (HP and LP) was divided into two treatments, M or V, resulting in 4 groups (HPM, HPV, LPM, LPV) distributed over 4 tanks with a total of ~1200 fish per tank. During this period, a marine diet with a high EPA and DHA content (21.4 %) and a plant-based diet with a low EPA and DHA content (1.9 %) were administered twice daily via automatic feeders (Arvo-Tec, Sterner). The 49-week period between the nutritional stimulus and the transfer to SW was referred to as intermediate FW phase. During this period, the fish were fed with commercial feed and uneaten feed was only recovered during the first 3 weeks after the stimulus. Nine weeks post stimulus phase, all fish were transferred to triplicated 800-l flow through tanks per group. From first feeding to 26 weeks a 24-h light regime was maintained and the water temperature was 11.0 ± 0.7 °C during stimulus and 12.6 ± 2.0 °C during intermediate phase respectively. When the fish reached approximately 60 g, after 26 weeks, they were placed in a simulated natural photoperiod (SNP) and transferred to flow through tanks with a volume of 1.6 m^3 until the end of the FW phase. During this time, a series of smoltification tests were carried out to ensure that the fish were fully adapted before transfer to SW, and water temperature was 4.7 ± 2.4 °C. After 26 weeks under SNP, approximately 1800 fish were transferred to SW pens to continue the experiment. Survival, oxygen levels and water temperature were monitored and recorded daily throughout the trial.

2.4.2. Seawater (SW)

The seawater phase was carried out at the Mowi test facilities in Ardnish, Scotland. During this time, all groups were fed twice daily for 1.5 h with automatic feeders (Arvo-Tec, Sterner). The fish arrived in early May 2021 and were randomly allocated to 12 pens of 5 m × 5 m × 5 m each (4 groups in triplicate). During the first 13 weeks of acclimatization in SW, referred to as intermediate SW, the fish were fed a commercial marine diet. Thereafter, the 36 week challenge phase began and all fish were fed either a plant-based diet or a challenge diet (containing at least 3 % EPA + DHA) until the end of the experiment at the fish size of appox. 4 kg. Throughout the SW phase, the fish received a daily overfeeding of 15 % and uneaten feed was recovered automatically 30 min after the end of each feeding period. Fish were subject to ambient temperature (13.7 ± 2.6 °C during initial acclimatization and 10.6 ± 3.2 °C during challenge) and natural light regime throughout the seawater phase. Survival rate was recorded daily, in addition to oxygen level (106.0 ± 12.3 % saturation, 8.8 ± 0.3 mg/L during acclimatization and 96.2 ± 12.6 % saturation, 9.0 ± 1.4 mg/L during challenge), and salinity (33.66 ± 1.28 ‰ during acclimatization and 29.65 ± 2.89 ‰ salinity during challenge).

2.5. Sampling procedures

Fish were starved for 24 h before sampling and recording individual body weights (BW) of tank populations. Individual animals were randomly sampled and euthanized with an overdose of anaesthetic (tricaine, 1000 ppm; MS-222, Pharmaq, Norway; S1K) followed by a blow to the head. Total length and BW were recorded before individual fish were processed for further analyses. After each sampling point any underperforming fish was culled. At the end of the stimulus phase, indicated in Fig. 1 as T0, 30 fish per tank were collected and pooled in triplicate groups of 10 fish. A small incision was made in the stomach of each fish, to allow preservative penetration, then animals were added to a 60-mL Sterilin bottle (ThermoFisher Scientific; Loughborough, UK) containing 20 mL of RNALater® (ThermoFisher Scientific). At all other time points (T1, T2, and T3), the gastrointestinal tract (GIT) of 5 fish per tank was dissected and aseptically opened. Remaining digestive debris was washed from the GIT with sterile Hank's balanced salt solution (ThermoFisher Scientific) before swabbing mucus and mucosa from the anterior (downstream of the pyloric caeca) and distal intestine. The swabs were placed into 2-mL Nunc screw-cap cryovials (ThermoFisher Scientific) containing 600 µL of RNALater®. Samples were refrigerated prior to analysis.

The specific growth rate (SGR) was calculated following the formula by Ricker (1975), SGR (%) = 100 x (eg - 1), here g = (ln (BWf) – ln (BWi)) / D. BWf and BWi correspond to final and initial body weight respectively, and D is the number of days of feeding. Feed efficiency (FE) or the ratio between feed consumption and fish weight, was estimated as (BWf - BWi)/FI, where FI is the feed intake.

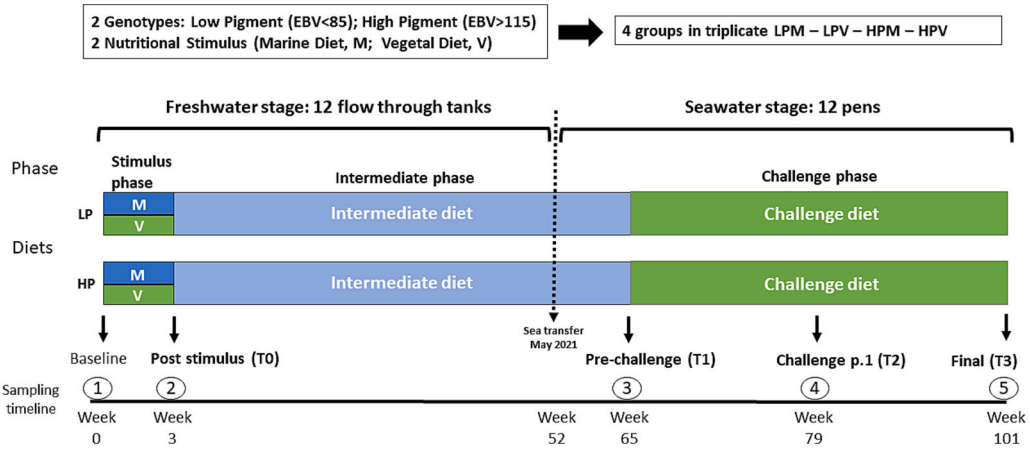

Fig. 1. Trial design plan.

2.6. Gut and feed microbiota analysis

2.6.1. Bacterial DNA extraction

The DNA extraction procedure has previously been described by Rimoldi et al. (2020) using the DNeasy PowerSoil® Pro Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions with an additional mechanical lysis step. Briefly, DNA was extracted from 300 μL of mucosal bacterial suspension and 200 mg of each feed (three aliquots/feed). Due to size limitations, the entire abdominal section of the larval fish was processed after removal of the head and caudal fin following stimulus (T0). The concentration and purity of extracted DNA was measured spectrophotometrically using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Milan, Italy) then stored at −20 °C until the preparation of the NGS library.

2.6.2. Illumina NGS library preparation

The 16S amplicon sequencing library was prepared using the GalSeq srl sequencing service (Milan, Italy) according to the Illumina protocol "16S Metagenomic Sequencing Library Preparation for Illumina MiSeq System" (#15044223 rev. B, https://support.illumina.com). The composition of the bacterial communities was determined by sequence analysis of the hypervariable region V4 of the 16S rRNA gene, which was amplified using using the oligonucleotides 515F: GTGYCAGCMGCCGCGGTAA and 806R GGACTACNVGGTWTCTAAT (Rimoldi et al., 2021). Libraries were sequenced on a MiSeq system (Illumina) using a paired-end 2 × 250 bp sequencing strategy.

2.6.3. Raw sequencing data analysis

The raw sequencing data were analysed using the QIIME 2 ™ (version 2020.2) pipeline (Bolyen et al., 2019) with the SILVA database (https://www.arb-silva.de/) used to complete the taxonomic assignment of amplicon sequence variants (ASVs). The entire data analysis pipeline included a pre-processing step in which the paired-end sequencing reads were trimmed for adapters, quality filtered ($Q > 30$) and merged. Then the remaining high-quality reads were then dereplicated and singletons and chimeric sequences were removed using the QIIME DADA2 denoise-paired command. The output of the DADA2 step was a table recording the number of ASVs observed for each sample. Next, taxonomic classification was performed at the genus level and eukaryotic, mitochondrial and chloroplast ASVs were removed. Alpha (within a single sample) and beta (between samples) diversity of bacterial communities was performed using the QIIME commands for alpha and beta phylogenetics, respectively.

For alpha diversity, Chao 1, Shannon and Simpson indices were calculated. For beta diversity, weighted and unweighted UniFrac distances were calculated depending on whether relative abundance or only presence/absence was considered. The UniFrac distances of the individual samples were visualised using two-dimensional principal coordinate analyses (PCoA) (Lozupone and Knight, 2005; Lozupone et al., 2007).

To visualise the core microbiota (ASVs present in at least 2/3 of the samples per diet/batch group), a Venn diagram was created using the Venny 2.1 tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.6.4. Predictive functional analysis of bacterial communities

The software package PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) was used to predict the functional profile of microbial communities using 16S rRNA marker gene sequences and the Greengenes (v.13.8) reference database. Metagenomic functions and pathways were predicted using KEGG pathways (Kanehisa et al., 2023; Kanehisa and Goto, 2000). The PICRUSt output files were then run using the Statistical Analysis of Metagenomic Profiles (STAMP) v 2.1.3 software package (Parks et al., 2014), with expanded error plots generated for each comparison.

2.7. Statistical analyses

Statistical analyses for growth parameters were completed using IBM SPSS Statistics for Macintosh software, Version 28.0 (Armonk, NY: IBM Corp, USA). Data were tested for normality with a Shapiro-Wilk test and homogeneity of variances using the Levene's test. All datasets without a normal distribution were log and square root transformed before re-evaluation. A non-parametric route using a Kruskall-Wallis test with Dunn's post-hoc test with Bonferroni correction was applied when data were not normally distributed. Similarly, in datasets where homogeneity of variance was not confirmed a Welch's test was used, followed by a Games-Howell post-hoc test. For normally distributed data, a two-way ANOVA was performed considering stimulus (S), genotype (G) and their interaction (S*G), followed by a one-way ANOVA to compare means between the four groups which, when significant, continued with Tukey's post hoc test, significance was accepted at $p < 0.05$.

Data on alpha diversity of gut samples (mucosa) were analysed using a two-way ANOVA with diet and sample origin as independent factors. A non-parametric PERMANOVA test with 999 permutations was applied to assess differences in beta diversity between groups, while the remaining microbiota data, microbiota relative abundances and metabolic pathways, were analysed by two-way ANOVA and Welch's two-tailed *t*-test, respectively. All statistical analyses for analysing taxonomic profiles were performed using PAST v3 software (Hammer et al., 2001), with significance set at $p < 0.05$.

3. Results

3.1. Growth performance and feed utilisation

Survival was high in all tanks (>99 %; Table 2) during SW and genotype was the primary determinant of growth differences before and during the challenge. In this sense, IBW in the intermediate SW phase was greater in HP fish than in LP fish ($p < 0.001$; Table 2). The fish from the HPM group had significantly higher IBW than the LPM ($p = 0.006$; Table 2) and LPV ($p = 0.026$; Table 2) groups, while the fish from the HPV group were significantly heavier than the LPM group ($p = 0.022$; Table 2). The SGR was significantly higher in the LP groups than in the HP groups ($p < 0.001$; Table 2) and between the LPM fish compared to HPM ($p = 0.004$; Table 2) and HPV ($p = 0.008$; Table 2).

Genotype was also the most important factor after 14 weeks exposure to the challenge diet (T1 to T2), with significantly higher FBW ($p = 0.004$), SGR ($p = 0.009$) and FE ($p = 0.035$) in LP groups compared to HP (Table 2). In addition, LPV fish exhibited significantly higher FBW compared to HPM and HPV treatments ($p < 0.031$; Table 2). There were no other statistically significant differences ($p > 0.05$; Table 2) in terms of growth parameters during this period. As observed in the previous phase, genotype was the main determinant of the differences observed during the last 22 weeks of exposure (T2 to T3). In agreement with the FBW results observed at the end of T2, IBW was significantly higher in LP fish than in HP fish ($p = 0.008$) and the LPM and LPV groups were significantly larger than the HPM and HPV groups ($p < 0.042$; Table 2). However, SGR shifted to significantly greater values in HP compared to LP fish ($p < 0.001$) and HPM and HPV versus LPM and LPV groups ($p < 0.014$), during this period (Table 2). There were no statistically significant differences ($p > 0.05$; Table 2) between FBW, survival, and FE for each of the periods.

3.2. Gut microbiota analysis

3.2.1. Sequencing efficiency

The analysis of the microbiota by next-generation sequencing was performed separately on gut and feed samples from T0 (post-stimulus), T1 (pre-challenge), T2 (mid-challenge point) and T3 (end of the feeding trial). The number of high-quality reads, taxonomically classified according to the Silva database excluding chloroplasts and mitochondria,

Table 2
Growth parameters and feed efficiency during the seawater phase.

Genotype	High Pigment				Low Pigment						
NS	Marine		Vegetable		Marine		Vegetable		*p*		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	S	G	S*G
SW transfer to T1											
IBW (g)	132.0^{a}	4.9	126.2^{ab}	3.7	103.7^{c}	5.5	110.2^{bc}	1.6	ns	<0.001	ns
FBW (g)	403.0	19.4	397.2	1.7	404.6	2.0	399.0	16.9	ns	ns	ns
Survival (%)	98.9	0.2	99.6	0.2	98.9	0.8	98.4	1.2	ns	ns	ns
SGR (%/d)	1.3^{b}	0.0	1.3^{b}	0.0	1.5^{a}	0.0	1.4^{ab}	0.0	ns	<0.001	ns
FE	0.6	0.0	0.7	0.0	0.8	0.1	0.7	0.1	ns	ns	ns
T1 to T2											
IBW (g)	439.7	13.7	431.4	6.2	438.3	4.5	441.8	18.0	ns	ns	ns
FBW (g)	1467.9^{b}	46.1	1473.4^{b}	12.9	1577.5^{ab}	26.8	1603.8^{b}	26.2	ns	0.004	ns
Survival (%)	94.9	0.6	97.2	1.0	95.2	1.9	95.6	2.4	ns	ns	ns
SGR (%/d)	1.4	0.0	1.4	0.0	1.5	0.0	1.5	0.0	ns	0.009	ns
FE	1.0	0.0	1.0	0.0	1.1	0.1	1.1	0.0	ns	0.035	ns
T2 to T3											
IBW (g)	1527.7^{b}	52.3	1506.9^{b}	18.3	1613.5^{a}	24.2	1646.7^{a}	23.9	ns	0.008	ns
FBW (g)	4137.9	228.9	4049.7	62.6	3916.8	60.0	3892.2	33.3	ns	ns	ns
Survival (%)	99.2	0.4	99.2	0.4	99.1	0.9	98.8	0.0	ns	ns	ns
SGR (%/d)	0.7^{a}	0.0	0.7^{a}	0.0	0.6^{b}	0.0	0.6^{b}	0.0	ns	<0.001	ns
FE	1.0	0.1	0.9	0.0	0.8	0.0	0.9	0.0	ns	ns	ns

IBW, initial body weight; **FBW**, final body weight; **Surv**, survival; **SGR**, specific growth rate; **FE**, feed efficiency. Significance was estimated for nutritional stimulus (**S**), genotype (**G**) and their interaction (**S*G**) with a two-way ANOVA and was accepted at $p < 0.05$. Differences between treatments were assessed using a one-way ANOVA with a Tukey's post hoc test. A Welch's test with Games-Howell post hoc test was performed in parameters where homogeneity of variance was not confirmed. Independent-Samples Kruskal-Wallis and Dunn's Test was performed for parameters without a normal distribution. Significant differences between treatments are represented by different letters.

was 295,621; 774,673; 1,339,683; and 704,528 for groups T0, T1, T2 and T3, respectively. Good coverage of greater than 0.99 was achieved for all four NGS analyses, indicating that the ASVs detected are representative of the entire microbial communities. All raw sequencing data have been submitted to the public European Nucleotide Archive (EBI ENA), access code: **PRJEB73592**.

3.2.2. *Alpha and beta diversity*

The alpha diversity of the gut samples (Additional file 1) showed that the Chao1, Simpson and Shannon indices at T0 were influenced by

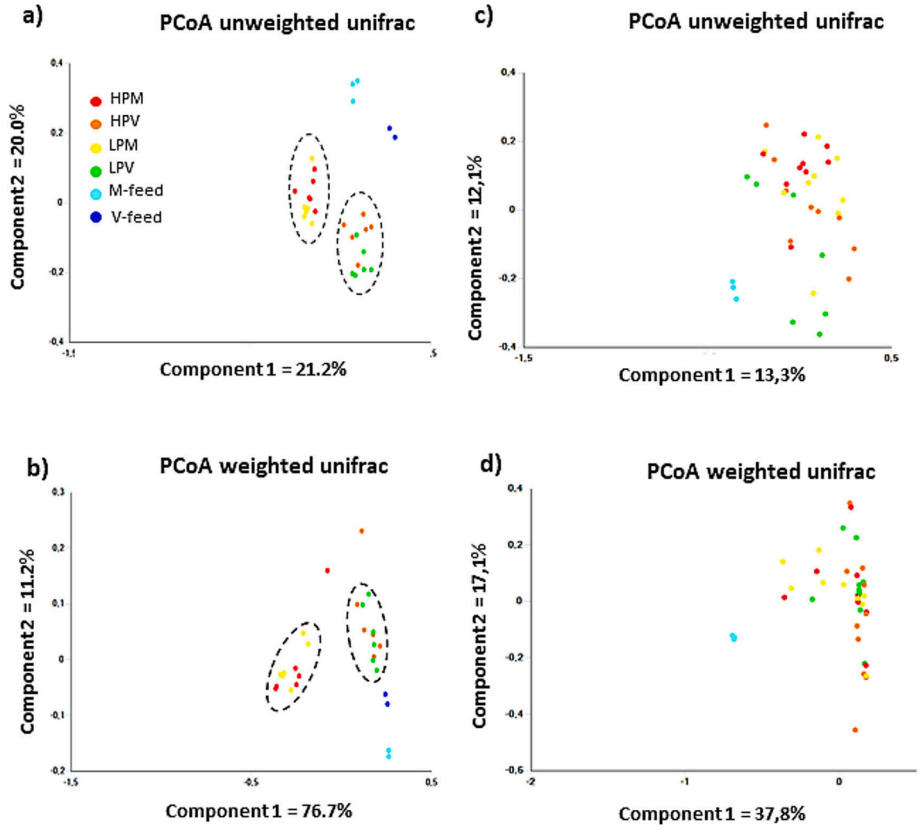

Fig. 2. Principal coordinate analysis (PCoA) of (a, c, d) unweighted and (b) weighted UniFrac distances of gut mucosa and feed-associated microbial communities at genus level at time of sampling: (a, b) T0, (c) T1, (d) T2.

stimulus diet and were higher in larvae fed a plant-based diet. In contrast, alpha diversity indices were not influenced by diet or genotype in the T1 and T3. At the challenge midpoint T2, only the Chao1 species richness index was influenced by diet and was higher in fish fed a marine diet, regardless of genotype. The sequencing depth for the calculation of alpha diversity indices was set to 8333; 6667; 19,000 and 17,777 reads for groups T0, T1, T2 and T3, respectively.

Beta diversity analysis revealed an overall effect of genetic batch and/or diet on the microbial community profiles of end stimulus T0 samples, which were clustered separately in both the unweighted and weighted UniFrac PCoA analyses (Fig. 2a, b). At the T1 and T2 timepoints the separation between the communities was less pronounced, with some overlap between the experimental groups and significant differences between the bacterial communities only in the unweighted UniFrac distances (Fig. 2c, d). In contrast, at the end of the feeding trial (T3), no differences were observed between the bacterial communities of gut in terms of beta diversity (data not shown). Multivariate permutation analysis using the PERMANOVA test with 999 permutations confirmed the PCoA results (Additional file 2).

3.2.3. Feed-associated bacterial communities

46,000 high-quality sequences were obtained from the T0 feed samples and correctly assigned down to genus level. Characterisation of the microbial community profiles revealed significant differences between the marine and plant feeds. In contrast, no differences were found in species richness (Chao-1 index) and biodiversity (Shannon and Simpson index) (data not shown). Regardless of the type of feed, the bacterial communities associated with the feed consisted of 4 main phyla (Fig. 3a), 6 classes, 9 orders, 18 families and 18 genera (Fig. 3b). The complete microbial profiles of the feeds can be found in the Additional file 3.

At the phylum level, the M diet had a higher proportion of Proteobacteria (62 %) than the V diet (24 %). In contrast, the V diet was characterised by a higher proportion of Firmicutes (75 %) than M (28 %) (Fig. 3a). According to Welch's two-sided *t*-test, a total of 18 bacterial genera differed significantly between the two diets (Table 3). In particular, the genera *Actinomyces*, *Enterococcus*, *Oceanobacillus* and *Photobacterium* were associated with the M-feed. In contrast, the plant-based feed revealed an association with some genera notable for probiotic properties, including *Lactobacillus*, *Weisella*, and *Leuconostoc*.

Firmicutes (52 %) and Proteobacteria (46 %) mainly formed the microbial profile of the M diet used in the intermediate phase T1. At the genus level, *Lactobacillus* (41 %), *Leuconostoc* (25 %) and *Lactococcus* (13 %) were the most abundant genera. Similarly, Firmicutes (73 %) and Proteobacteria (24 %) represented more than 90 % of the feed-associated bacterial taxa in the plant challenge diet (T2 and T3

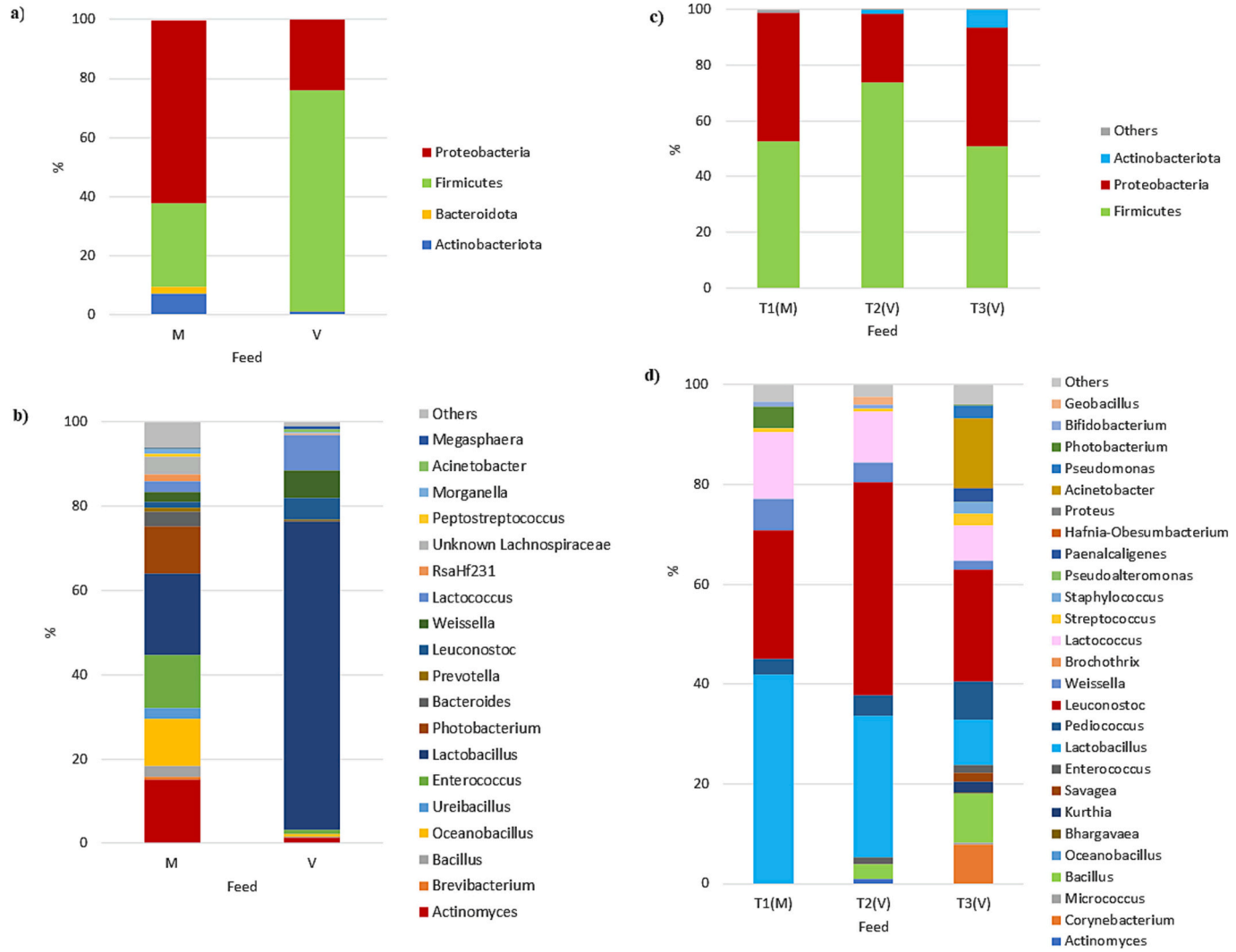

Fig. 3. Mean relative abundance (%) of the most abundant bacteria in feeds used during the stimulus phase (T0) at phylum (a) and genus (b) level ($N = 3$), and during the phases T1, T2, and T3 at phylum (c) and genus (d) level ($N = 3$). Only bacteria with a total abundance of $\geq$0.5 % were reported. Bacteria with lower abundance were summarised in a group indicated as "Others".

Table 3
List of bacteria and their relative abundance at different phylogenetic levels found in the larvae at the end of the stimulus phase (T0) and which differed between the test groups. Significance was estimated for nutritional stimulus (**S**), genotype (**G**) and their interaction (**S*G**) with a two-way ANOVA. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.0001$; (**ns**) $p \geq 0.05$.

	HPM (%)	SD (%)	HPV (%)	SD (%)	LPM (%)	SD (%)	LPV (%)	SD (%)	*Sig.*		
									G	S	S*G
PHYLUM											
Spirochaetota	75.69	23.06	9.28	3.83	80.50	11.44	8.15	2.29	ns	***	ns
Firmicutes	6.08	2.92	56.59	19.12	5.85	2.00	60.48	15.35	ns	***	ns
Proteobacteria	12.70	15.86	23.10	14.22	7.05	4.15	21.81	15.43	ns	**	ns
CLASS											
Brevinematia	75.69	23.06	9.28	3.83	80.50	11.44	8.15	2.29	ns	***	ns
Bacilli	5.60	2.68	55.03	19.09	5.37	1.80	59.14	15.32	ns	***	ns
Gammaproteobacteria	12.50	15.95	23.02	14.25	6.79	4.09	21.59	15.18	ns	*	ns
ORDER											
Vibrionales	0.96	0.45	0.02	0.03	1.07	0.26	0.02	0.05	ns	***	ns
Lactobacillales	1.99	1.14	45.95	14.65	2.09	1.09	55.14	14.47	ns	***	ns
Veillonellales-Selenomonadales	0.01	0.01	1.03	0.66	0.05	0.05	0.90	0.51	ns	***	ns
Brevinematales	75.69	23.06	9.28	3.83	80.50	11.44	8.15	2.29	ns	***	ns
Pseudomonadales	6.12	9.47	18.77	15.11	1.35	0.67	15.59	15.45	ns	**	ns
FAMILY											
Vibrionaceae	0.96	0.45	0.02	0.03	1.07	0.26	0.02	0.05	ns	***	ns
Leuconostocaceae	0.21	0.17	7.31	2.13	0.23	0.19	9.79	3.06	ns	***	ns
Lactobacillaceae	0.85	0.53	33.01	11.28	0.80	0.49	38.82	9.78	ns	***	ns
Brevinemataceae	75.69	23.06	9.28	3.83	80.50	11.44	8.15	2.29	ns	***	ns
Streptococcaceae	0.22	0.14	4.87	1.16	0.21	0.14	5.50	1.85	ns	***	ns
Veillonellaceae	0.01	0.01	0.96	0.62	0.05	0.06	0.82	0.45	ns	***	ns
Pseudomonadaceae	0.11	0.10	7.38	10.15	0.31	0.32	8.28	16.81	ns	*	ns
Moraxellaceae	6.01	9.46	11.39	5.51	1.04	0.50	7.31	4.66	ns	**	ns
GENUS											
Alkanindiges	0.00	0.00	0.03	0.06	0.00	0.00	6.32	4.47	ns	ns	***
Photobacterium	0.83	0.37	0.00	0.00	0.93	0.31	0.00	0.00	ns	***	ns
Weissella	0.13	0.14	3.94	1.27	0.08	0.11	5.46	1.95	ns	***	ns
Leuconostoc	0.09	0.07	3.37	1.09	0.15	0.14	4.34	1.14	ns	***	ns
Lactobacillus	0.84	0.52	32.97	11.25	0.80	0.49	38.72	9.81	ns	***	ns
Lactococcus	0.22	0.15	4.66	1.05	0.18	0.12	5.18	1.73	ns	***	ns
Brevinema	75.69	23.06	9.28	3.83	80.50	11.44	8.15	2.29	ns	***	ns
Megasphaera	0.01	0.01	0.95	0.62	0.05	0.06	0.80	0.45	ns	***	ns
Acinetobacter	0.15	0.22	11.20	5.46	0.19	0.16	0.71	0.45	***	***	***
Pseudomonas	0.11	0.10	7.38	10.15	0.31	0.32	8.28	16.81	ns	*	ns
[Agitococcus]_lubricus_group	5.76	9.49	0.15	0.24	0.64	0.34	0.27	0.28	ns	*	ns
Arcicella	0.14	0.19	0.08	0.13	1.14	1.09	0.84	0.85	**	ns	ns
Pedobacter	0.55	0.92	0.01	0.01	1.31	1.14	0.19	0.15	*	*	ns

sampling points). At the genus level, the lactic acid bacteria *Leuconostoc* (42–22 %), *Lactobacillus* (28–9 %) and *Lactococcus* (10–7 %) were the most representative genera together with *Bacillus* (9 %) and *Acinetobacter* (13 %) (Fig. 3c, d).

3.2.4. Host-associated bacterial communities

3.2.4.1. Post stimulus phase T0. Irrespective of the stimulus diet, the larval gut microbiota consisted of 12 phyla, 16 classes, 43 orders, 69 families and 101 genera (Additional file 4). However, when only the most representative taxa (relative abundance higher than 0.5 %) were considered, the microbial profile consisted of 5 phyla (Fig. 4a), 6 classes, 11 orders, 17 families and 21 genera (Fig. 4b).

When plotted on a Venn diagram, the common core microbiota consisted of 19 genera. (Fig. 5). At the phylum level, Spirochaetota and Proteobacteria accounted for more than 90 % of the reads found in M-diet larvae, regardless of genotype. In contrast, Firmicutes and Proteobacteria were the most abundant phyla in larvae fed the V diet (Fig. 4a). Analysis by two-way ANOVA revealed that diet was the main factor influencing microbiota profiles. An effect of genotype was found only at the genus level, while an interaction between stimulus diet and genotype (S*G) was significant in only one case. In particular, dietary stimulus had a significant effect on the relative abundance of the phyla Firmicutes, Proteobacteria and Spirochaetota. The latter was mainly represented by the class Brevinematia, being enriched by the M diet ($p < 0.01$), while the other two phyla, mainly represented by the classes Bacilli and Gammaproteobacteria, were more abundant in larvae fed the V diet ($p < 0.001$) (Table 3). Accordingly, at the family level, the families Lactobacillaceae, Leuconistocaceae, Streptococcacae, and Veillonellaceae, which all belong to the Firmicutes phylum, and the families Moraxellaceae and Pseudomonadaceae, belonging to the Gammaproteobacteria, were enriched in the V-diet-fed larvae. In contrast, larvae fed the M diet had a higher abundance of the Brevinemataceae and Vibrionaceae families, regardless of genotype (Table 3). Significant differences in relative abundance based on genotype were observed for the genera *Arcicella* and *Pedobacter*, both of which were associated with a low pigment genotype. A significant interaction (S*G) was found for the genus *Acinetobacter*, which was more abundant in HPV larvae (Table 3). All the other genera, which occurred with different frequencies in the experimental groups, were only influenced by the nutrition factor. The lactic acid bacteria representing genera *Lactobacillus*, *Leuconostoc*, *Weisella* and *Lactococcus* were positively influenced by the V diet ($p < 0.001$), while the microbiota of the larvae fed the M diet consisted mainly of the genus *Brevinema* (75–80 %) (Fig. 4b). In addition, the genus *Photobacterium* was only detectable in HPM and LPM larvae (Table 3).

3.2.4.2. Pre-challenge phase T1. In the gut mucosal samples collected at time T1, 21 phyla, 33 classes, 92 orders, 148 families and 238 genera (Additional file 5) formed the overall microbial profile, independently of genotype and diet. However, when considering only the most representative taxa (frequency higher than 0.5 %), the composition of the gut microbiota was reduced to 5 phyla (Fig. 6a), 7 classes, 17 orders, 29 families and 30 genera (Fig. 6b). In these samples, no central member of

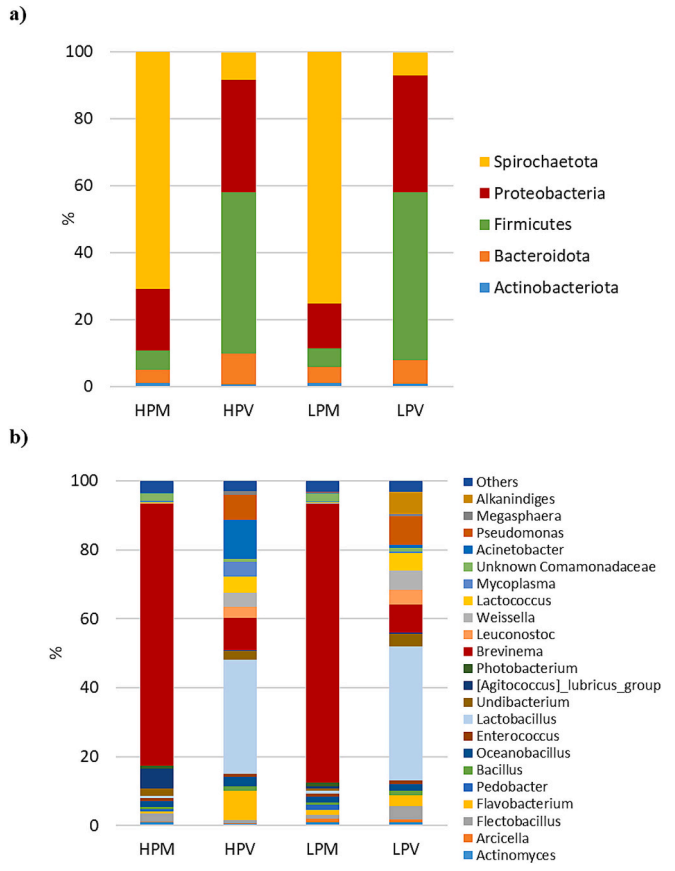

Fig. 4. Mean relative abundance (%) of the most abundant bacteria in the larvae during the stimulus phase (T0) at phylum (A) and genus (B) level ($N = 6$). Only bacteria with a total abundance of ≥0.5 % were reported. Bacteria with lower abundance were summarised in a group labelled "Others".

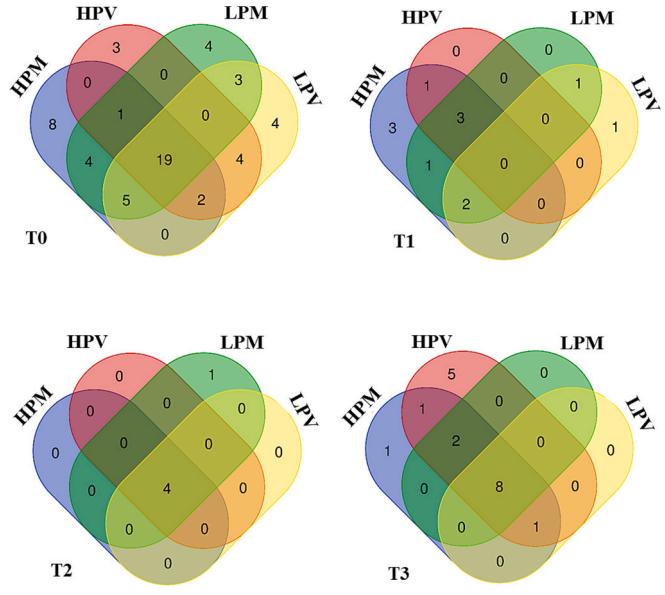

Fig. 5. Venn diagrams showing the comparison of unique and shared genera between different experimental groups and sampling timepoints T0, T1, T2 and T3.

the gut microbiota was detected at the genus level (Fig. 5).

At the phylum level, Proteobacteria (40–70 %) and Firmicutes (22–33 %) were the most abundant bacteria in all experimental groups,

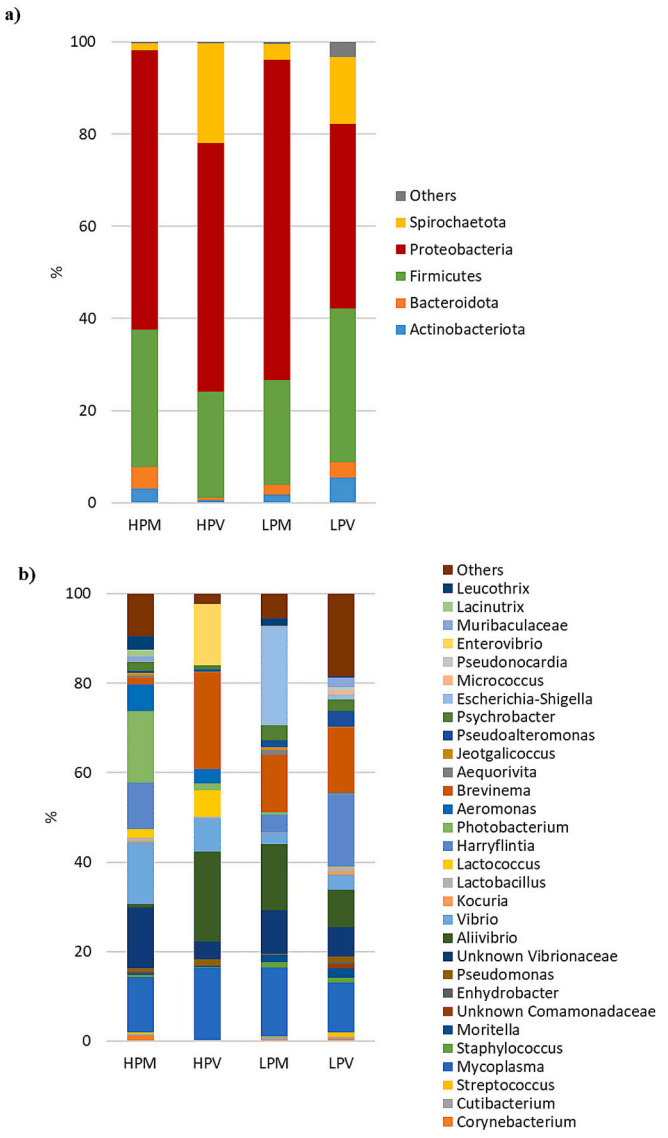

Fig. 6. Mean relative abundance (%) of the most abundant bacteria in the intestinal mucosa during the pre-challenge phase T1 at phylum (a) and genus (b) level ($N = 9$). Only bacteria with a total abundance of ≥0.5 % were reported. Bacteria with lower abundance were summarised in a group labelled "Others".

regardless of diet and genotype. In salmon fed a plant-based diet at the larval stage, the Spirochaetota phylum was also highly represented in the gut (Fig. 6a). However, the two-way ANOVA analysis revealed no differences between the relative abundances of the bacteria at this taxonomic level.

At the order level, however, the Peptostreptococcales-Tissierellales were influenced by genotype, as they were more abundant in salmon with low pigment genotype (Table 4). An effect of the first feeding was found for the family Flavobacteriaceae and the genus *Aequorivita*, both of which were enriched in M fish, while the genus *Photobacterium* was associated with the HP genotype (Table 4).

3.2.4.3. Challenge phase T2. The combined microbiota of the intestinal mucosa of fish during challenge phase T2 comprised 5 phyla, 7 classes, 15 orders, 23 families and 29 genera (Additional file 6). However, the most representative taxa were assigned to 3 phyla (Fig. 7a), 4 classes, 7 orders, 8 families and 9 genera (Fig. 7b).

As shown in the Venn diagram, the bacterial core microbiota comprised four genera (Fig. 7). At the phylum level, Proteobacteria and

Table 4
The list of bacterial gut taxa and their relative abundance (mean ± SD) that differed between the experimental groups in phase T1. Significance was estimated for nutritional stimulus (**S**), genotype (**G**) and their interaction (**S*G**) with a two-way ANOVA. (*) $p < 0.05$; (**ns**) $p \geq 0.05$.

	HPM (%)	SD (%)	HPV (%)	SD (%)	LPM (%)	SD (%)	LPV (%)	SD (%)	*Sig.*		
									G	S	S*G
ORDER											
Peptostreptococcales-Tissierellales	0.05	0.10	0.02	0.04	0.79	1.37	0.38	0.66	*	ns	ns
FAMILY											
Flavobacteriaceae	3.15	6.54	0.10	0.15	2.08	2.72	0.35	0.59	ns	*	ns
GENUS											
Aequorivita	0.41	1.05	0.02	0.04	1.05	1.61	0.00	0.00	ns	*	ns
Photobacterium	16.00	24.83	1.59	2.55	0.54	1.60	0.05	0.14	*	ns	ns

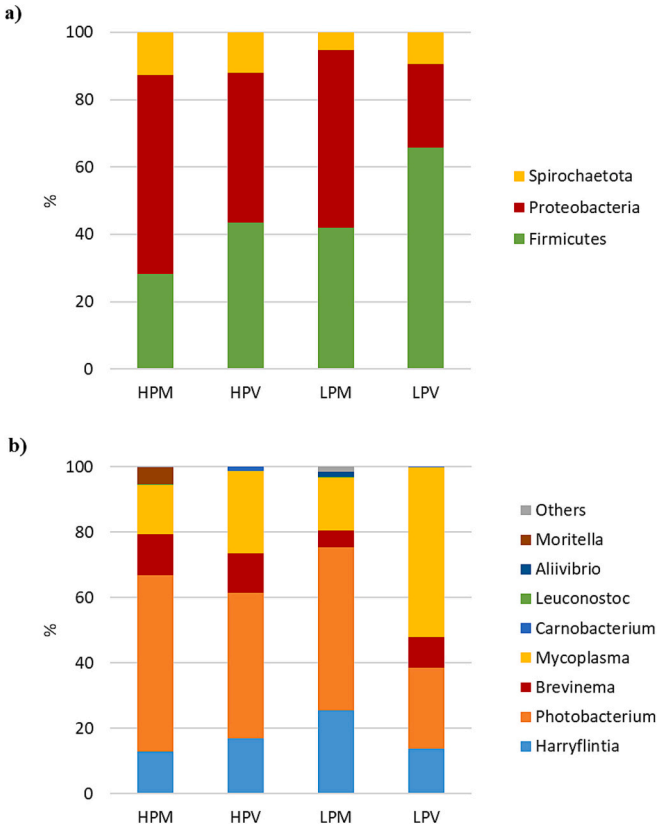

Fig. 7. Mean relative abundance (%) of the most abundant bacteria in the intestinal mucosa during the challenge phase T2 at phylum (a) and genus (b) level (N = 9). Only bacteria with a total abundance of ≥0.5 % were reported. Bacteria with lower abundance were summarised in a group labelled "Others".

Firmicutes accounted for almost 90 % of the reads, while the Spirochaetota phylum was between 9 and 12 %. (Fig. 7a). Differences in the relative abundance of taxa were found at the order, family and genus level. In particular, the families Leuconostocaceae and Carnobacteriacea, which are mainly represented by the genera *Leuconostoc* and *Carnobacterium*, were influenced by the stimulus diet, as they were more abundant in the M or V groups, respectively (Table 5). An interaction effect between stimulus diet and genotype was found for the genus *Aeromonas*, which belongs to the order Aeromonadales and the family Aeromonadaceae, which was only detected in the LPM fish group (Table 5).

3.2.4.4. Final phase T3. At the end of the feeding trial, the whole gut microbiota consisted of 10 phyla, 13 classes, 32 orders, 39 families and 57 genera (Additional file 7). Considering only the most representative taxa (relative abundance ≥0.5 %), 3 phyla (Fig. 8a), 3 classes, 5 orders, 17 families and 17 genera (Fig. 8b) accounted for more than 99 % of the total microbial community. Of these, the phylum Tenericutes, mainly represented by the genus *Mycoplasma*, was dominant in all experimental groups (72–95 %). The final gut samples had a common core microbiota consisting of 8 genera (Fig. 5). At this stage of sampling, two-way ANOVA of taxa abundance data revealed no influence of diet or genotype (data not shown).

3.2.5. Predictive functional analysis of bacterial communities

A predictive functional analysis of the bacterial communities colonising the larvae revealed differences only in the response to the stimulus diet, with no effect due to genotype. Regardless of genotype, the first feeding with the V diet led to an increase in gene copies involved in photosynthesis and transporter pathways. In contrast, the M diet correlated with flagellar assembly, ribosome biogenesis and aminoacyl-t-RNA biosynthesis (Fig. 9). The functional composition of the microbial communities in the gut before the challenge phase (T1) and at the end of the feeding trial showed no differences between the experimental groups. In contrast during challenge phase T2, three bacterial metabolic pathways, including purine metabolism, aminoacyl-t-RNA biosynthesis and the ribosome, differed significantly in fish with low pigment genotype (Fig. 10).

Table 5
The list of bacterial taxa and their relative abundance (mean ± SD) in the gut that differed between the experimental groups in phase T2. Significance was estimated for nutritional stimulus (**S**), genotype (**G**) and their interaction (**S*G**) with a two-way ANOVA. (*) $p < 0.05$.

	HPM (%)	SD (%)	HPV (%)	SD (%)	LPM (%)	SD (%)	LPV (%)	SD (%)	*Sig.*		
									G	S	S*G
ORDER											
Aeromonadales	0.00	0.00	0.00	0.00	1.28	3.13	0.00	0.00			*
FAMILY											
Leuconostocaceae	0.18	0.49	0.00	0.00	0.13	0.20	0.01	0.01		*	
Aeromonadaceae	0.00	0.00	0.00	0.00	1.28	3.13	0.00	0.00			*
Carnobacteriaceae	0.00	0.00	1.26	3.12	0.00	0.00	0.22	0.60		*	
GENUS											
Leuconostoc	0.17	0.46	0.00	0.00	0.12	0.18	0.01	0.01		*	
Aeromonas	0.00	0.00	0.00	0.00	1.28	3.13	0.00	0.00			*
Carnobacterium	0.00	0.00	1.26	3.12	0.00	0.00	0.22	0.60		*	

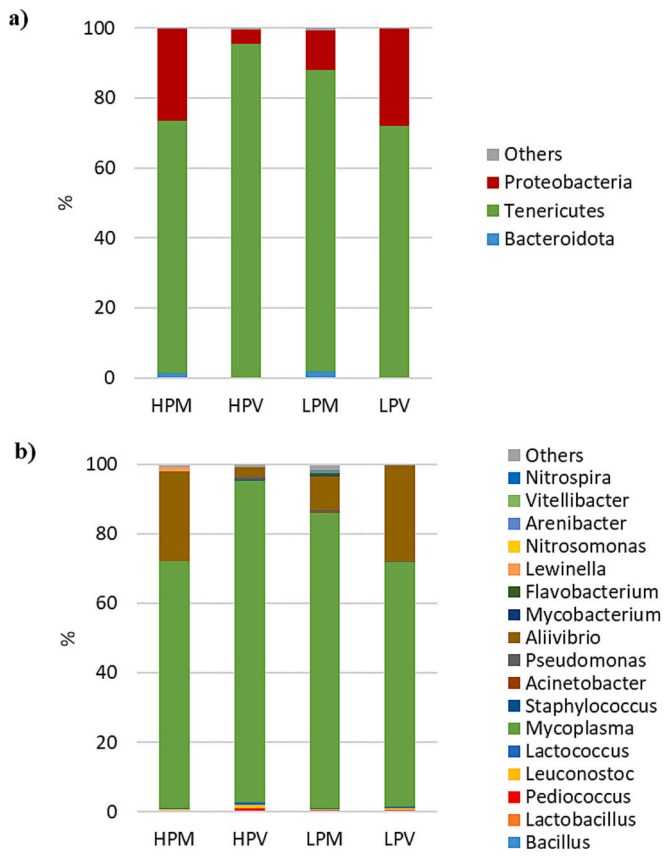

Fig. 8. Mean relative abundance (%) of the most abundant bacteria in the intestinal mucosa at the end of the study (T3) at phylum (a) and genus (b) level (N = 6). Only bacteria with a total abundance of ≥0.5 % were reported. Bacteria with lower abundance were summarised in a group labelled "Others".

4. Discussion

Nutritional programming (NP) has been studied in various species that are important for aquaculture (Geurden et al., 2014; Lage et al., 2018; Turkmen et al., 2019). Among other things, the long-term effects on growth (Øie et al., 2017), nutrient metabolism (Vagner et al., 2007) and the use of plant-based feed (Lazzarotto et al., 2016) have been investigated. In previous experiments investigating NP in salmonids, in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) respectively, fish that were initially fed a plant-based "stimulus" diet showed higher growth compared to a marine control diet when the same fish were later switched back to the plant-based diet in a challenge phase (Clarkson et al., 2017; Geurden et al., 2013). However, in both prior studies, the challenge phase was completed early in development, prior to SW transfer in the case of salmon, and without isogenic lines. Thus, the overall aim of the present study was to investigate the long-term impact in Atlantic salmon post SW transfer and the interaction with different high and low pigmentation genotypes, following a first feeding NP stimulus. In particular, this study investigated the effects of NP on gut microbial community composition and any corresponding relationship with growth and feed utilisation outcomes.

In this current study, the stimulus diet had no significant influence on the growth parameters and feed utilisation during the "challenge" phase in seawater. This is despite the observations at the end of the stimulus phase in FW, where the V-stimulated groups sustained lower SGR, FE and final body weight compared to the M-stimulated fish. This indicates that the effects observed in freshwater (lower SGR, FE and final body weight) were not transferable to seawater conditions. In contrast, previous studies revealed positive short-term effects of NP on growth and nutrient utilisation in salmonids, but no evidence was found that this mechanism persisted long-term in this current study, indicating a possible attenuation of the NP effect over time (Clarkson et al., 2017; Geurden et al., 2013; Tawfik et al., 2024). For instance, our performance results at the end of the freshwater phase contrast with Clarkson et al. (2017), where FE was better in fish stimulated with a plant-based diet at first feeding compared to fish fed a M-diet. This earlier study described physiological adaptations at the level of gene expression in the plant-stimulated groups after the "challenge", which may have improved nutrient utilisation and increased the fish's tolerance to plant-based diets (Vera et al., 2017). The fact that NP did not increase post-challenge growth and FE in the current study may indicate a weakening of the NP effect over time, which is consistent with previous studies examining NP through first exogenous feeding of Atlantic salmon using a V-diet (Tawfik et al., 2024). Therefore, further research is required to investigate whether fish performance can respond favourably to a plant-based diet in seawater and whether a booster or "stimulus" at a different developmental stage produces a longer lasting effect.

In contrast to NP, the pigmentation genotype appeared to be the driving factor for changes in growth and feeding parameters during the challenge phase of our experiment in SW. This is consistent with previous findings where selection for a single trait influenced other important traits, suggesting complex interactions between genetic selection and physiological responses (Brezas and Hardy, 2020; Naya-Català et al., 2022). It is important to note that the fish were selected for pigmentation, as this is a key trait of economic importance in Atlantic salmon (Garber et al., 2019), which could be altered by the fatty acid profile in plant-based diets (Bjerkeng et al., 1997). A previous study by Naya-Català et al. (2023), combining NP and genetic selection, analysed the offspring of gilthead sea bream from selected (for growth) and non-selected broodstocks programmed with a low FM and FO diet. As expected, the offspring from the selected families maintained significantly higher growth parameters than the non-selected ones. In contrast to results from this current trial, the offspring exposed to a challenge diet (low FM and no FO) had significantly lower final body weight and greater SGR and FCR compared to groups fed the control diet (Naya-Català et al., 2023).

Toomey et al. (2020) found that selection for a single trait can affect the expression of other important traits. Thus, as observed in our study, fish selected for HP also showed low growth rates during the challenge phase. Regardless of this, the observed shift in growth parameters between genotypes could indicate underlying mechanisms or interactions that need to be further investigated. A study revealed that rainbow trout from three isogenic lines sustained similar growth when fed a marine diet but developed differently after exposure to either a marine or a plant-based diet five months after the initial feeding (Callet et al., 2021). This could be due to the different FE between the groups. Furthermore, when analysing the transcriptome profiles in the liver, it was found that the different genotypes activate different signalling pathways relating to nutrient metabolism (Callet et al., 2021). Nevertheless, further studies are needed as, to our knowledge, the interaction between nutritional stimulus and genotype has not yet been described in Atlantic salmon.

Associations between growth parameters and gut microbiota were found in Atlantic cod (*Gadus morhua*) and mangrove killifish (*Kryptolebias marmoratus*) (Forberg et al., 2016). Trinh et al. (2017) demonstrated that cod larvae with low and high SGR showed significant differences in bacterial community profiles at 4 out of 10 sampling sites between 7 and 42 days post-hatching (dph). However, in their study, the influence of age correlated more strongly with the differences in the composition of the larval microbiota up to 28 days post-hatching than SGR. Accordingly, in the present study, species richness, species diversity and dynamic changes in gut microbiota composition were associated with fish age, irrespective to NP and pigmentation genotype. As previously observed in gilthead seabream, the Chao1 species richness index progressively decreased over the fish production cycle (Naya-Català et al., 2022). Lokesh et al. (2019) investigated the progressive transition of bacterial communities of Atlantic salmon from birth to adulthood and

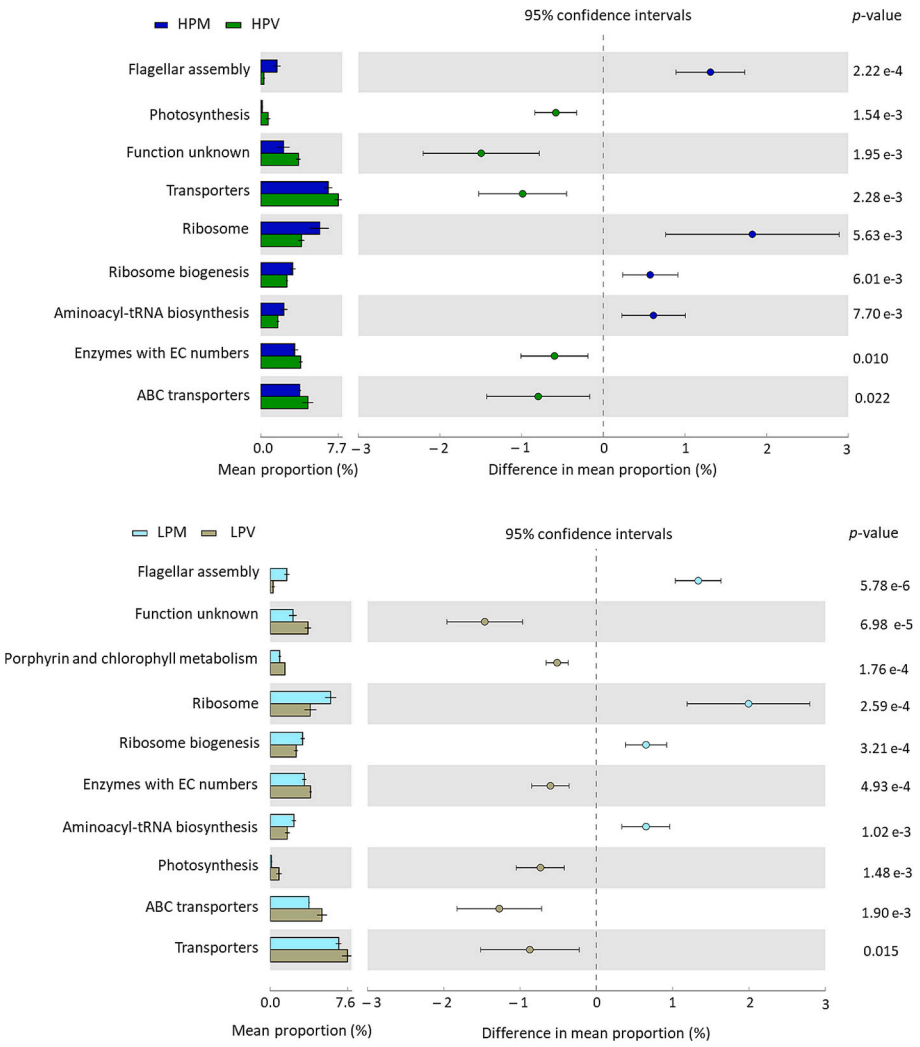

Fig. 9. Predictive functional analysis of bacterial communities (PICRUSt) of group T0.

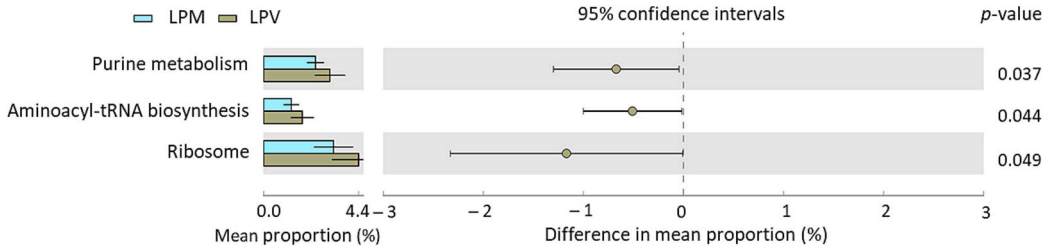

Fig. 10. Predictive functional analysis of bacterial communities (PICRUSt) in group T2.

found that the number of common species (Shannon index) of bacterial communities was significantly lower in the 44-week post-hatching (wph) phase compared to the 20-week phase. In the same study, differences were found between the 20-week stage and later stages (44 and 62 weeks) in terms of the weighted unifrac distances of the bacterial communities. Similarly, the gut microbiota of zebrafish (*D. rerio*) changed in both diversity and composition over the lifespan, with alpha and beta diversity decreasing throughout development (Stephens et al., 2016; Xiao et al., 2021).

Furthermore, the available data indicate that the first feeding with a soybean meal-based diet did not significantly affect the gut microbiota of larval largemouth bass (*Micropterus salmoides*), but only age-related changes in the gut microbiota of this species were documented (Kwasek et al., 2021).

Although fish developmental stage appears to be a crucial factor in shaping the gut microbiota (Lokesh et al., 2019), it is worth noting that in this present study, the gut region investigated (whole larvae in the stimulus phase compared to whole gut in the intermediate and challenge phase) may also have contributed to the differences in microbiota between T0 and subsequent samplings.

Previously, a plant protein-based diet was fed to zebrafish (*D. rerio*) broodstock as an alternative maternal transfer NP strategy investigating the effect on the gut microbiota of the offspring, but no changes were found (Kwasek et al., 2022).

In the current trial, the diet in the early life phase had a significant effect on the beta diversity of the bacterial communities in the gut of the

fish. At the end of the stimulus phase, there was a significant difference in community structure in response to diet, regardless of pigmentation genotype. The differences in beta diversity decreased with age until they disappeared in adulthood. As a result, the core microbiota tended to be more stable over time, with changes likely due to environmental factors rather than early life factors such as NP.

Consistent with the alpha and beta diversity metrics, the plant diet influenced the relative abundances of the most common phyla between groups at the end of the stimulus phase, including reducing the number of harmful microbes and promoting the growth of beneficial bacteria in the salmon larvae. Regardless of genotype, Firmicutes dominated the microbiota profile of salmon larvae fed a plant-based diet. In particular, at the genus level, the plant diet significantly increased the relative abundance of the lactic acid bacteria (LAB) *Lactobacillus*, *Leuconostoc*, *Weisella* and *Lactococcus*, which are commonly used as probiotics in fish (Torres-Maravilla et al., 2024). In agreement with our results, bacterial genera belonging to the orders Lactobacillales and Bacillales were specifically promoted when plant proteins were included in the diet of juvenile rainbow trout (Ingerslev et al., 2014; Michl et al., 2017). In general, the increase in LAB is beneficial for maintaining fish health during early development and beyond (Gatesoupe, 2008; Ringø et al., 2018).

LAB can stimulate the host's immune system and produce antimicrobial compounds that can control colonisation by fish pathogens (Araújo et al., 2015; Askarian et al., 2011; Ringø et al., 2018). The increase in LAB is perhaps not surprising, as the higher proportion of indigestible fibre in soybean and other plant foods could explain the higher abundance of these microorganisms, which are known to use such substrates for their metabolism and growth (Wang et al., 2021) and the high abundance of LAB in the digestive tract of salmonids fed a plant-based diet has been reported previously (Desai et al., 2012; Gajardo et al., 2017).

In a recent study on rainbow trout, we found that Firmicutes and Proteobacteria levels are different relating to diet, with fish fed a plant-based diet having a higher Firmicutes:Proteobacteria ratio than those fed animal-based diets (Rimoldi et al., 2018), and this has also been confirmed in other carnivorous fish, including non-programmed Atlantic salmon (Gajardo et al., 2017; Serra et al., 2021). In contrast, the marine stimulus diet significantly promoted the Spirochaetota phylum, which is mainly represented by the genus *Brevinema*. In a previous pompano (*Trachinotus ovatus*) feeding trial, *Brevinema* was associated with reduced growth rates and abundance was negatively correlated with butyric acid concentration, a clear indication of a negative effect of phylum Spirochaetota on fish immunity and gut health (Zhang et al., 2023). Butyrate, the esterified form of butyric acid, is one of the short chain fatty acids, bacterial metabolites that are produced by fermentation in the gut, linked to improved health and immunity (Tan et al., 2014). The differences observed in terms of relative abudance of specific genera can be also explained by the different lipid source in the diet. Previous studies suggested an association of some gut bacteria, including *Brevinema*, with the source of dietary oil. For instance, in gilthead sea bream the increase in *Brevinema* can be linked to the use of DHA-rich algae oil as a main FO replacer (Naya-Català et al., 2021, 2022). Actually, the marine stimulus diet had ten times more DHA content than plant-based diet.

However, as already mentioned, in this study the differences in growth post-stimulus were mainly due to the genotype, with a higher SGR in the LP group. A genotype effect in microbial population was observed at the genus level, with an increase of *Arcicella* and *Pedobacter* associated with the genotype of low pigmentation, while an interaction between genotype and stimulus diet was found for the genus *Acinetobacter*. It has previously been reported that *Pedobacter* is predominant in the gut of healthy Atlantic salmon (Wang et al., 2018). In contrast, the genus *Acinetobacter*, which was enriched in HPV salmon larvae, includes potential opportunistic pathogens of fish, such as *A. lwoffii*, *A. junii*, *A. pittii*, *A. baumannii* and *A. johnsonii* (Bi et al., 2023; Malick et al., 2020).

Bacterial communities appeared to be mainly determined by the diet fed at the time of sampling and NP did not induce any permanent changes. In fact, the differences in the composition of the microbiota decreased drastically during the intermediate or challenge phases until they disappeared at the end of the experiment. Similarly, the first feeding diet did not programme the gut microbiota of trout fry, which was also modulated according to the current diet fed at point of sampling (Michl et al., 2019; Michl et al., 2017). This evidence suggests that the gut microbiota is influenced more by the current diet than by a previous nutritional intervention.

At the end of the pre-challenge phase, a significant genotype effect was observed for the genus *Photobacterium*, whose relative abundance was higher in HP salmon. Regardless of genotype, M-fish gut microbiota was enriched with the Flavobacteriaceae family, represented by the genus *Aequorivita*. Interestingly, an NP effect was observed at the beginning of the challenge phase. First feeding history influenced the relative abundance of three genera with *Carnobacterium* abundant (>1 %) in V-fish, while *Leuconostoc* and *Aeromonas* were found in small quantities in association with the diet of the M-stimulus. *Carnobacterium* species are part of the LAB and several studies in recent decades have shown that they can act as probiotics in fish (Ringø, 2024).

The presence of LAB depends on the content of fermentable substrates in the host's gut, which were likely more abundant in the stimulus V compared to M diet. Therefore, the observed enrichment of the genus *Carnobacterium* could indicate an improved utilisation of the plant food in the programmed fish. However, we are aware that this result is not sufficient to prove beyond doubt that there is a link between NP and an altered gut microbiota for two reasons.

Firstly, microbial changes did not persist until the end of the challenge phase and secondly, because the feed stimulus had no significant effect on feed utilisation during the "challenge" phase in seawater. Accordingly, previous studies using 16S rRNA sequencing have shown no significant association between NP with plant feed and fish gut microbial profiles (Kwasek et al., 2022, 2021; Michl et al., 2019, Michl et al., 2017; Patula et al., 2021).

As expected, the prediction of the microbial metabolic pathways revealed significant differences in the composition of the metabolic potential in response to the stimulus diet principally at the end of the stimulus phase. However, the metabolic pathways involved were not directly involved in energy and nutrient metabolism, consistent with the lack of differences observed in fish growth and conversion rate. Interestingly, more copies of genes coding for flagellar assembly were present in the M fish regardless of genotype. The pathway of flagella formation could play an important role in the adhesion process of motile and potentially pathogenic bacteria such as Spirochaetota, which predominantly form the bacterial community in the gut (Li et al., 2021; Strnad et al., 2024). Indeed, Spirochaetota are known for their high motility and chemotactic attraction to mucin, which permit them to penetrate the mucus and associate with the intestinal mucosa. It is well known that the microbiota associated with mucosal surfaces may have a more significant impact on influencing the host's metabolic processes. For instance, in salmon there was a correlation between mucosa-associated genus *Brevinema andersonii* and the expression in the distal intestine of genes related to immune responses and barrier function (Li et al., 2021).

5. Conclusions

Based on the results presented, several conclusions can be drawn about the effects of NP and pigmentation genotype on growth, feed utilisation and gut microbiota of Atlantic salmon.

The study found no significant effect of the early plant diet (stimulus) on growth parameters and feed utilisation during the challenge phase in seawater.

In contrast, pigmentation genotype had a stronger influence on growth and feeding parameters during the seawater "challenge" phase.

The HP genotypes had lower growth rates, suggesting that genetic selection for pigmentation may influence other traits such as growth performance.

However, the composition of the gut microbiota changed significantly with fish age, independent of NP and pigmentation genotype. The early diet (plant-based) influenced beta diversity and the relative abundance of certain bacteria (e.g., increased lactic acid bacteria), but these differences diminished over time and did not persist into adulthood.

Regardless, certain microbial genera were associated with different genotypes and diets, indicating possible genotype-diet interactions that need to be further investigated.

In conclusion, although early nutritional programming with a plant-based diet can transiently alter growth parameters and gut microbiota composition in Atlantic salmon, these effects do not persist into the seawater phase. Pigmentation genotype plays a more important role in long-term growth performance. The gut microbiota is primarily influenced by the age of the fish and the current diet rather than by early nutritional interventions. Further studies are needed to explore the interactions between genotype, stimulus diet and microbiota and to determine whether alternative NP strategies could have more sustainable effects.

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CRediT authorship contribution statement

Simona Rimoldi: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Karla Fernandez Quiroz:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Violeta Kalemi:** Formal analysis, Data curation. **Stuart McMillan:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Ingunn Stubhaug:** Resources. **Laura Martinez-Rubio:** Resources. **Mónica B. Betancor:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Genciana Terova:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The authors declare that all data are present in the manuscript

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Appendix A. Supplementary data

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