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Intestinal microbiota shifts by dietary intervention during extreme heat summer episodes in farmed gilthead sea bream (*Sparus aurata*)

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ABSTRACT

Climate change and its associated extreme events alter a number of physiological processes that also affect the homeostatic relationship of the host with their microbial communities. The aim of this study was to gain more insights on this issue, examining the effect of the record breaking-heat summer of 2022 on the gut microbiota of farmed gilthead sea bream (*Sparus aurata*), reared from May to August at the IATS research infrastructure (Spain's Mediterranean coast). Fish were fed daily with four experimental diets, containing two different lipid levels (16 % and 14 %) with/without a commercial emulsifier (0.1 %; Volamel Aqua, Nukamel). On August 9th, concurrently with the historical record of water temperature (30.49 ºC), fish were sampled for analysis of blood-stress markers and water/intestinal microbiota. Gut microbiota analysis clearly evidenced the increased abundance of bacteria of Spirochaetota phylum, mainly represented by the genus *Brevinema*. This microbiota shift was not driven by environmental colonization as this bacteria genus remained residual in water samples with the increase of temperature. Bayesian network and functional enrichment analyses suggested that the high abundance of *Brevinema* exploits and negatively enhances a condition of imbalance in intestinal homeostasis, which was almost completely reversed by the use of dietary emulsifiers in combination with low energized diets. This phenotype restoration occurred in concomitance with changes in circulating levels of cortisol and glucose. Altogether this highlights the potential use of *Brevinema* as a heat-stress indicator, reinforcing the value of dietary intervention as a valuable solution to mitigate the negative impact of global warming on aquaculture production.

1. Introduction

Aquaculture is the fastest growing food production sector over recent decades with an average annual growth rate of 6.7 % (FAO, 2022a). However, to guarantee the sustainability of the sector, the aquaculture industry needs to face widespread environmental challenges, including the adaptation of the production systems to climate change (Ahmed et al., 2019; FAO, 2022b). Global warming is in fact a main concern for the future of aquaculture, which is reinforced by the role of temperature as a master abiotic factor in ectotherm organisms (Islam et al., 2022; Reid et al., 2019; Volkoff and Rønnestad, 2020). Otherwise, while ocean warming poses a worldwide issue, the Mediterranean Sea is particularly vulnerable to temperature fluctuations because of its geographical location and semi-enclosed nature (Sakalli, 2017). Certainly, over the last 40 years, the rise of the Mediterranean surface temperature was more than three times higher than the registered increase in open oceans (IPCC, 2023; Pisano et al., 2020). Besides this, the occurrence of extremely warm episodes, defined as marine heat waves, becomes more and more frequent making the nearshore Mediterranean aquaculture highly vulnerable to climate change (Atalah et al., 2024; Dayan et al., 2023; Hamdeno and Alvera-Azcaráte, 2023).

At the organismal level, there is now increasing evidence that shifts in intestinal microbial communities merit special attention in a context of global warming. Precise and consistent information regarding the effect of heat-stress episodes on intestinal microbiota are still limited and sometimes contradictory. Indeed, most of the discrepancies derived from the duration and severity of the heat stress, culture system and developmental stage at which heat stress is induced (Diwan et al., 2023; Xavier et al., 2024; Zhou et al., 2023). In any case, gut microbiota actively participates in the regulation of numerous fish physiological

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processes, including growth, nutrient digestibility, disease resistance and stress response by means of a complex network of interactions within and among microbial populations (Brown et al., 2019; Butt and Volkoff, 2019; de Bruijn et al., 2017; Lopez Nadal et al., 2020; Ou et al., 2021). Thus, according to the hologenome theory of evolution, the holobiont (host-microbiome system) would act as a unit of evolutionary selection, facilitating the fast genomic changes of the microbiota and the adaptation of the holobiont to constantly changing environmental conditions (Simon et al., 2019). In this way, it must be noted that fish gut microbiota not only changes with sex and age (Piazzon et al., 2019; Zhao et al., 2020), but also with season and host genetics that ultimately shapes the microbial community to cope with changes in diet composition (Naya-Català et al., 2022; 2024; Piazzon et al., 2020) and other stressors including thermal stress. Beneficial effects in the modulation of the intestinal bacterial composition were also achieved using feeding probiotics in a wide range of animal including fish, and also pigs and broilers (Ciji and Akhtar, 2021; Moroni et al., 2021; Ringseis and Eder, 2022; Naiel et al., 2022). This strategy has in fact been proven to re-establish the physiological conditions of the microbiota and to enhance productivity, health, and welfare even under heat stress conditions.

At the cellular level, both warm- and cold-blooded organisms deal with thermal stress through different mechanisms (Tattersall et al., 2012). Unlike mammals and birds, fish, given their inability to regulate internal temperature, can cope with up to a certain degree of heat stress mainly by adapting growth rates and basal activity (Islam et al., 2022). However, although with different intensities and speed of reaction, numerous physiological stress response processes are shared between endotherms and ectotherms animals. For all organisms, heat stress is intertwined with oxidative stress; therefore, the first common strategy is the increase of production of antioxidants, due to the activation of brain centres and the consequent release of catecholamines and corticosteroids (Madeira et al., 2013; Alfonso et al., 2021; Alsharif, 2022). Indeed, several dietary modifications focusing on feed additives with antioxidant activity have been considered in broiler chickens to buffer and/or prevent the excess production of reactive oxygen species (ROS) (Emami et al., 2021). Another important metabolic response which appears as a common compensatory feature across species is the suppression of feed intake. In the endotherm animals this physiological change is related with the reduction of the endogenous heat production due to digestion and absorption of feed (Onagbesan et al., 2023). In fish, instead, this mechanism seems to serve to stem further oxidative stress. Thermal stress in fact leads to an energy imbalance and low digestive efficiency, so a continuous food intake during high temperature would result in an over-production of ROS (Volkoff and Rønnestad, 2020). Whilst in aquatic organisms the processes that determine changes in appetite, due to temperature increase, are not fully understood, in poultry and other species such regulatory action is also accompanied by the increase of hepatic lipogenesis that enhances lipid deposition rates in spite of the reduction of feed intake (Guo et al., 2021; Hao et al., 2016; Lan et al., 2022; Yin et al., 2021). In this context, sustained heat stress can compromise hepatic function by promoting hepatic steatosis. To counter this phenomenon, in poultry the use of natural and synthetic emulsifiers have been successfully applied as a nutritional therapy to alleviate the negative impact of heat stress (Yin et al., 2021). This action is supported, at least in part, by the pleotropic action of bile acids upon gut microbiota and the enterohepatic circulation system (Schubert et al., 2017; Sun et al., 2020). Likewise, there is now evidence in farmed barramundi (*Lates calcarifer*) that high fat diets reduced fish tolerance to extreme water temperatures (Gomez Isaza et al., 2019). Moreover, natural or synthetic emulsifiers improve growth and lipid digestion in a wide range of fish, including gilthead sea bream (Ruiz et al., 2023a, 2023b). However, it remains uncertain and poorly documented how we can monitor the extent to which dietary intervention can contribute to alleviate the negative impact of heat stress in gilthead sera bream. Thus, the aim of this study was to evaluate in a highly cultured Mediterranean fish the combined effect of dietary fat level and a commercial emulsifier (Volamel Aqua, Nukamel) on gut microbiota during the record-breaking summer of 2022 at the Spanish Mediterranean coast. To further understand the physiological significance of the achieved results, data on gut microbiota were used to model and unravel the causal relationships of bacterial populations using a Bayesian-Network (BN) approach (Soriano et al., 2023). The hypothesis of work is that the predictive modelling of changes in gut microbiota helps to better understand the impact of thermal stress in aquaculture productivity and sustainability, giving support to the design of effective remediation strategies to mitigate the negative impact of climate change on animal production.

2. Material and methods

2.1. Ethics statement

All the procedures received approval from the Ethics and Animal Welfare Committee of the Institute of Aquaculture Torre de la Sal (IATS), the CSIC Ethics Committee (with the authorization number 1295/2022), and the Generalitat Valenciana (under the license number 2022-VSC-PEA-0230). These procedures were conducted at the registered aquaculture infrastructure facility of IATS (facility code ES120330001055), adhering strictly to the guidelines set forth in the European Animal Directive (2010/63/EU) and the Spanish legal framework (Royal Decree RD53/2013) for the protection of animals used in scientific experiments.

2.2. Animals

Juveniles of gilthead sea bream (*Sparus aurata*) were purchased in April 2022 (5 g approximate mean body weight) from a commercial Mediterranean hatchery (Piscimar, Burriana, Spain), being acclimatized to the IATS-CSIC research infrastructure for five weeks under natural photoperiod and temperature conditions at the IATS latitude (40° 5′N; 0° 10′E). During all the trial, fish were maintained in an open flow-through system, ensuring oxygen content of water effluents above 85 % saturation and unionized ammonia below 0.02 mg/L.

2.3. Diets

Four isoproteic, plant-based diets containing 6 % fish meal were formulated for this study and produced by Research Diet Services (RDS, the Netherlands). The diet named HFD (High-Fat Diet) was formulated as a control diet mimicking the typical proximate composition of commercial aquafeeds, including a fat content of 16 %. Based on this formulation, three experimental diets were designed to incorporate the two variables studied: reduced fat content (14 %) and the addition of a commercial hydrophilic emulsifier (1000 ppm; Volamel Aqua, Nukamel, Belgium). The resulting diets were named as follows: High-Fat Diet (HFD), High-Fat Diet + Emulsifier (HFD-EMS), Low-Fat Diet (LFD), and Low-Fat Diet + Emulsifier (LFD-EMS) (Table 1).

2.4. Experimental setup and sample collection

After the acclimation period, randomly selected fish of 12.48 ± 0.25 g body weight were distributed in twelve 500 L tanks (3 tanks/diet; 45 fish/tank). Fish were fed with the experimental diets by hand once a day at a fixed time (12 a.m.) until visual satiety over the course of the trial (11 weeks from May to August). During the first half of the trial (46 days), emulsifier supplementation supported a 10 % improvement of feed conversion ratio (FCR) with the increase of water temperature from 20ºC to 25ºC. Such improvement was masked during the second half of the trial with the achievement of the historical record of water temperature (30.49ºC, August 9th, 2022) at our latitude (Fig. S1). At this time, water samples were taken for analyses of microbiota, and 12 fish per diet (4 fish per tank) were anaesthetized with 0.1 g/L of tricaine-methanesulfonate (MS-222, Sigma-Aldrich, St. Louis,

Table 1
Formulation and proximate composition of experimental diets used in the feeding trial.

Ingredients (%)	HFD	HFD-EMS	LFD	LFD-EMS
Sunflower meal	14.84	14.84	14.33	14.33
Vital Wheat Gluten	10.90	10.90	10.78	10.78
Wheat flour	10.50	10.50	12.97	12.97
Soybean meal 48	10.00	10.00	10.00	10.00
Soy protein concentrate	8.25	8.25	8.25	8.25
Corn gluten	8.00	8.00	8.00	8.00
Poultry meat meal	8.00	8.00	8.00	8.00
Rapeseed oil	6.61	6.61	6.54	6.54
Haemoglobin meal	7.00	7.00	7.00	7.00
Fish meal 999 LT	6.00	6.00	6.00	6.00
Fish oil	6.00	6.00	4.00	4.00
Lecithin	1.00	1.00	1.00	1.00
Vitamin Mineral Premix[a]	1.00	1.00	1.00	1.00
Lysine	1.00	1.00	1.00	1.00
Limestone	0.78	0.68	1.00	0.90
Monocalcium phosphate	0.12	0.12	0.12	0.12
Volamel Aqua		0.100		0.100
Proximate composition (%)				
Protein	47.0	47.0	47.0	47.0
Fat	16.0	16.0	14.0	14.0
Ash	6.4	6.4	6.4	6.4
Fiber	2.6	2.6	2.6	2.6
Moisture	7.0	7.0	7.0	7.0

[a] Premix (IU or mg / kg diet): Ascorbic acid phosphate, 160 mg; Biotin, 0.8 mg; Cholecalciferol, 2000 IU; Choline, 800 mg; Cobalt carbonate, 0.40 mg; Copper sulphate, 6 mg; Cyanocobalamin, 0.12 mg; Folic acid, 4 mg; Inositol, 240 mg; Iodate, 4 mg; Iron Sulphate, 80 mg; Menadione, 12 mg; Magnesium, 500 mg; Manganese sulphate, 24 mg; Niacin, 144 mg; Pantothenic acid, 40 mg; Pyridoxine, 20 mg; Retinol 10,000 IU; Riboflavin, 16 mg; Selenium selenite, 0.24 mg; Thiamine, 16 mg; Tocopherol, 240 mg; Zinc sulphate, 100 mg.

MO, United States) for blood and intestinal mucus sampling after a 48 hours fasting period. In order to have a reference of intestinal microbiota profile which can reflect the common bacterial distribution not affected by heat stress, fish with the same genetic background, same batch and fed with a commercial standard diet (Biomar, Palencia, Spain; EFICO 3053) were used. These fish were used as reference (REF), as they were sampled right before the extreme heat episodes during summer 2022, with water temperature below 25ºC.

Blood was taken from caudal vessels using heparinized syringes, and fish were rapidly sacrificed by cervical section. Plasma was obtained by centrifugation at 3000 × g for 20 min at 4°C, followed by storage of plasma aliquots at −80°C for later analysis. A portion of the anterior intestine (~2 cm) was opened and washed with sterile Hank's balanced salt solution before the scraping of intestinal mucus with the blunt edge of a sterile scalpel to collect the adherent intestinal microbiota. Mucus samples were then transferred to sterile Eppendorf tubes and maintained in ice until DNA extraction, immediately performed after sampling. To characterize water associated bacterial communities, 1 L of seawater was sampled from each of the experimental tanks using sterile glass bottles. Bacteria were then collected using a manifold filtration system with mixed cellulose esters filters with a pore size of 0.22 μm. Filters were transferred to individual sterile petri dishes and stored at −80ºC until DNA extraction.

2.5. Bacterial DNA extraction

DNA from adherent intestinal mucus microbiota (200 μL) was extracted using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions, including a previous lysis step with lysozyme (Sigma) at a concentration of 250 μg/mL for 15 min at 37ºC (Piazzon et al., 2019). DNA from water-associated bacterial communities was extracted using the purification kit DNeasy PowerSoil Pro (Qiagen). Filters were cut in small pieces and submitted to a mechanical lysis using the ceramic beads tubes provided in the kit, using FastPrep 24 homogenizer (MP Biomedicals) at 6 m/s for 30 s. Subsequent steps of the extraction were performed following the manufacturer's instructions. DNA concentration and quality in both cases were checked using NanoDrop 2000c (Thermo Fisher Scientific) and agarose gel electrophoresis (1 % w/v Tris-EDTA buffer). All DNA extracted samples were stored at −20ºC until sequencing.

2.6. Illumina Miseq sequencing of anterior intestine mucus

The V3-V4 hypervariable region of the 16S rRNA gene (341 F: 5' – CCTACGGGNGGCWGCAG – 3' 805 R: 5' – GACTACHVGGGTATC-TAATCC – 3') from adherent bacteria of intestine was sequenced with the Illumina MiSeq platform (2 ×300 paired-end run) at the Genomics Unit from the Madrid Science Park Foundation (FPCM, Spain) as described elsewhere (Piazzon et al., 2019). Raw sequenced data obtained was lodged in the Sequence Read Archive (SRA) under the Bioproject accession number PRJNA1137871 (BioSample accession numbers: SAMN42644020–67). Raw forward and reverse paired-reads were merged using VSEARCH v2.15.1 (Rognes et al., 2016), and then pre-processed using Prinseq v0.20.4 (Schmieder and Edwards, 2011). Sequences with > 5 % of N bases were discarded, and terminal N bases were trimmed in both ends of all sequences. Sequences with a length < 300 bp, with Phred quality score < 20 in both sequence ends or with an average Phred quality score < 25 were excluded. Individual sequences identified as amplicon sequence variants (ASVs) were aligned using Minimap2 v2.17-r941 (Li, 2021) with SILVA v138.1 (Yilmaz et al., 2014) as reference database.

2.7. ONT MinION sequencing of water microbiome

In order to monitor the microbiome composition of water in our facilities, ONT MinION device was used as a rapid and in-field sequencing platform. To do so, V1-V9 region of the 16S rRNA gene (27–1492 nt) from bacteria of water samples was amplified, and sequencing libraries were prepared using 16S Barcoding kit 1–24 (SQK-16S024). An input of 50 ng of DNA were used for the PCR amplification, using the LongAmp Hot Start Taq 2 × Master Mix (NEB, M0533S) and the primers provided in the kit (F: 5′ - ATCGCCTACCGTGAC - barcode - AGAGTTTGATCMTGGCTCAG - 3′ and R: 5′ - ATCGCCTACCGTGAC - barcode - CGGTTACCTTGTTACGACTT - 3′). Cycling conditions were 95 °C for 1 min, followed by 30 cycles of 95°C for 20 s, 52°C for 30 s, and 65°C for 2 min with a final extension step of 65 °C for 5 min (Toxqui-Rodriguez et al., 2023). After a clean-up step using AMPure XP Beads (Beckman Coulter), amplicons were quantified using PicoGreen dye (Thermo Fisher Scientific). Samples were pooled in 100 fmol pools and loaded in MinION devices using R9.4.1 Flow Cells (FLO-MIN106D). Raw sequenced data obtained was lodged in the Sequence Read Archive (SRA) under the Bioproject accession number PRJNA1137871 (BioSample accession numbers: SAMN42644068–79). Raw data were demultiplexed using MinKNOW v23.07.8 and basecalled using Guppy v7.0.8. The resulting FASTQ files were pre-processed using Porechop v0.2.4 (github.com/rrwick/Porechop) for barcode trimming, Nanofilt v2.8.0 (De Coster et al., 2018) for sequence length filtering, and Yacrd v0.6.2 (Marijon et al., 2020) for chimera detection and removal. Sequences identified as ASVs were taxonomical assigned using Minimap2 v2.17-r941 (Li, 2021) with SILVA v138.1 (Yilmaz et al., 2014) as reference database.

2.8. Blood stress markers

Circulating glucose and cortisol levels were measured in both cases from 10 μl of plasma obtained from the same individuals sampled for microbiota (n = 12). Glucose levels were assessed utilizing the Invitrogen™ Glucose Colorimetric Detection Kit (EIAGLUC, Invitrogen; sensitivity 0.413 mg/dL). Plasma cortisol levels were determined by

competitive ELISA assay using the Cortisol Immunoassay Kit (Ref. K003-H1W, Arbor Assays; sensitivity 27.6 pg/mL) according to the manufacturer's instructions.

2.9. Data analyses

Blood biochemical data were analysed by one-way ANOVA using SigmaPlot v14 (Systat Software Inc, United States). Normality of the data was verified by Shapiro-Wilk test, and Dunn's post-test was used for multiple comparisons among groups. Rarefaction curves, species richness estimates, and alpha diversity indices were obtained using phyloseq package for R (McMurdie and Holmes, 2013). Statistical differences in species richness and alpha diversity indices were determined by Kruskal-Wallis test using Dunn's post-test, with a significance threshold of $P < 0.05$. Beta diversity among groups was determined by permutational multivariate analysis of variance (PERMANOVA), using the non-parametric method *adonis* from the R package Vegan (Oksanen et al., 2015) with 10,000 random permutations. Differences in bacterial relative abundances at phylum level were tested using two-way ANOVA. To study detailed microbiota differences among groups, partial least-squares discriminant analysis (PLS-DA) was performed using EZinfo v3.0 (Umetrics Umeå, Sweeden). Hotelling's T^2 statistic was calculated and points above 95 % confidence limit were considered outliers and excluded from the model. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the model fit and prediction ability, respectively. The contribution to group separation of the different bacterial genus was determined by the variable importance in projection (VIP) value. VIP score > 1 was considered the threshold level to determine discriminant variables in the PLS-DA model (Kieffer et al., 2016; Li et al., 2012).

To study the intestinal bacterial interactions within the microbiota populations, a stochastic model, based on the construction of a comprehensive Bayesian Network (BN), was applied. For this purpose, the bacterial relative abundances at genera level of the four different groups (HFD; HFD-EMS; LFD; LFD-EMS) were merged and considered as input dataset for microbiota, while the lipid level and the presence/absence of the emulsifier in the diet were used as discrete experimental variables (Emulsifier & Lipid level). This model allowed the identification of the causal relationships between the set of variables and microbial taxa, defining a network hierarchy, by which the probabilistic dependence of one node to another is defined through a parent-child relationship. The build of the BN was performed using the on-line SAMBA tool (Structure-learning of aquaculture microbiomes using a bayesian approach) already described (Soriano et al., 2023). The SAMBA software used in this study was an updated version that makes feasible to identify clusters of nodes (bacteria) densely connected to each other, using the Leiden community detection method (Traag et al., 2019). The resulting clusters were then enriched with clusterProfiler 4.0 (Wu et al., 2021), using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways functional annotation protocol. The significance of the enrichment was calculated using a hypergeometric test, evaluating the differences between the number of OTUs within each cluster and the total number of OTUs (background, data not shown) involved in the pathways. Statistically significant values were then adjusted for multiple testing, using the Benjamini-Hochberg method to control the false discovery rate (FDR).

3. Results

3.1. Richness and diversity of gut microbiota

Illumina sequencing produced a total of 6679,185 high quality assigned reads for 48 gut samples with an average of 139,150 reads per sample. These reads were assigned to a total of 24,584 ASVs and a high percentage of them were classified up to genus level (85.7 %), and more than 95 % to the level of family (95.7 %), order (97.9 %), class (99.5 %) and phylum (99.9 %). Rarefaction analysis showed curves next to saturation (horizontal asymptote) (Fig. S2) and considering the number of assigned sequences an appropriate coverage of the bacterial community was achieved. Fig. 1 shows the richness (Chao1 and ACE) and diversity (Shannon and Simpson) indexes for the four experimental groups. Considering the Chao1 index, LFD-EMS fish showed a higher richness in comparison to fish fed non-supplemented diets regardless of fat level (HFD and LFD). The same fish showed a significant increase in microbiota richness in comparison to HFD when using the ACE estimator. In contrast, no differences in diversity indexes were found for any of the possible comparisons among groups.

3.2. Gut microbiota composition and discriminant analysis

Gut microbiota composition at phylum level is depicted in Fig. 2. The microbiota of fish with the same genetic background and not exposed to extreme summer temperatures (REF) was characterized by a predominance of the Proteobacteria phylum (> 70 %). However, Spirochaetota phylum appeared in high abundance in fish sampled during extreme heat episodes, becoming the most abundant phylum in HFD fish. Notably, a significant decrease in the relative abundance of this phylum was observed with both emulsifier supplementation ($P = 0.005$) and reduction of dietary fat level ($P = 0.011$). Conversely, Proteobacteria abundances increased significantly with lipid reduction ($P = 0.011$) and emulsifier addition ($P = 0.005$), gradually restoring the dominance of this phylum in the adherent microbiota. Additionally, the abundance of the Fusobacteria phylum increased significantly ($P = 0.033$) in the experimental fish fed with emulsifier supplemented diets (HFD-EMS, LFD-EMS).

To further investigate differences in bacterial composition, permutational multivariate analysis of variance was performed showing statistically significant differences among the four experimental groups (PERMANOVA, $F = 1.109$, $R^2 = 0.102$, $P = 0.047$). In order to study in more detail these differences, two PLS-DA models were constructed and statistically validated, driving the separation of each experimental variable, emulsifier supplementation (Fig. 3a) and lipid level (Fig. 3b). The model based on emulsifier addition (R2Y = 99 %, Q2 = 64 %) achieved a distinct separation between the EMS and non-EMS groups, with the first two principal components accounting for over 93 % of the total explained variance (Fig. S3a). The model based on dietary lipid level (R2Y = 98 %, Q2 = 56 %) also facilitated a clear separation, although slightly less pronounced than that observed with the emulsifier variable, with approximately 86 % of the total explained variance after two components (Fig. S3b). Both models were constructed using four components, as determined by cross-validation to avoid overfitting of the model. Validation of the models by random permutations can be found in Fig. S3c-d.

To determine the specific groups of bacteria driving the separation between experimental groups at a high level of confidence, only bacterial groups with a VIP value ≥ 1 and a relative abundance higher than 0.5 % were selected. This selection resulted in 11 and 12 taxa considered responsible for the separation based on emulsifier and lipid level variables, respectively. Fig. 4 shows the list of the 19 unique abundant taxa at genus level that drove the separation between groups in both models, seven specific of variable emulsifier model (*Pseudomonas, Thauera, Cetobacterium, Bacillus, Staphylococcus, Micrococcus* and *Streptomyces*) and eight from dietary lipid level (*Devosia*, *Paracoccus, Ralstonia, Clostridium, Brachybacterium, Cutibacterium* and *Blastococcus* genera, and *Rhizobiaceae* family). Additionally, four taxa were significantly discriminant in both models and, thus, their relative abundances were affected by both dietary variables (*Brevinema*, *Vibrio* and *Photobacterium,* and *Beijerinckiaceae*). Among them, *Brevinema* genus appeared as the most abundant bacterial taxa associated with heat-stressed fish in this study. Indeed, this unique genus represents almost the total contribution to Spirocheatota phylum (> 99 %), being the main responsible for the shifts observed at phylum-level with a wide range of abundance that

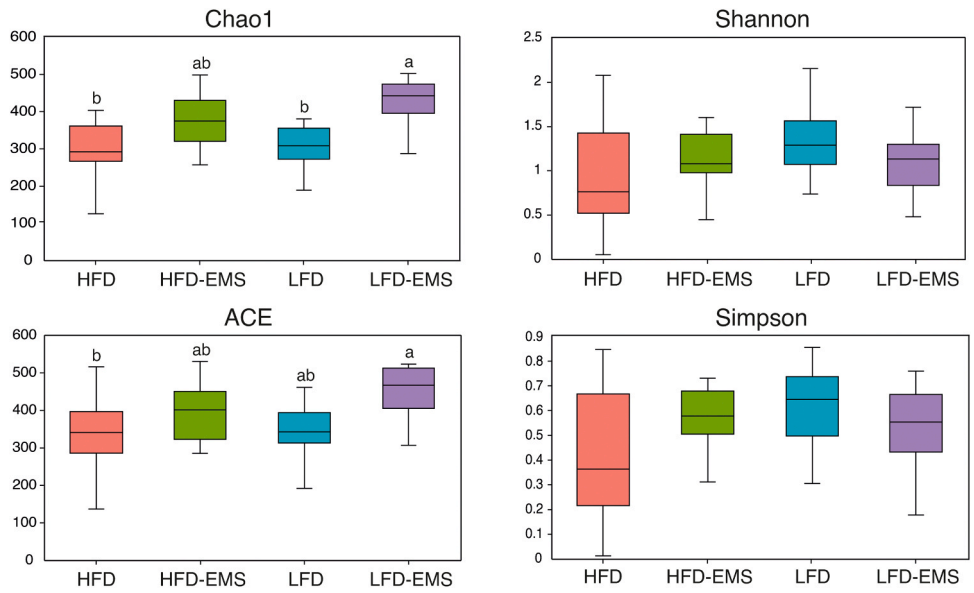

Fig. 1. Boxplots representing richness estimators (Chao1 and ACE) and diversity indexes (Shannon and Simpson) of the bacterial microbiota of anterior intestine of fish fed with experimental diets (n = 12). Different letters indicate significant differences among groups (Kruskal-Wallis test with Dunn's post-test, P < 0.05).

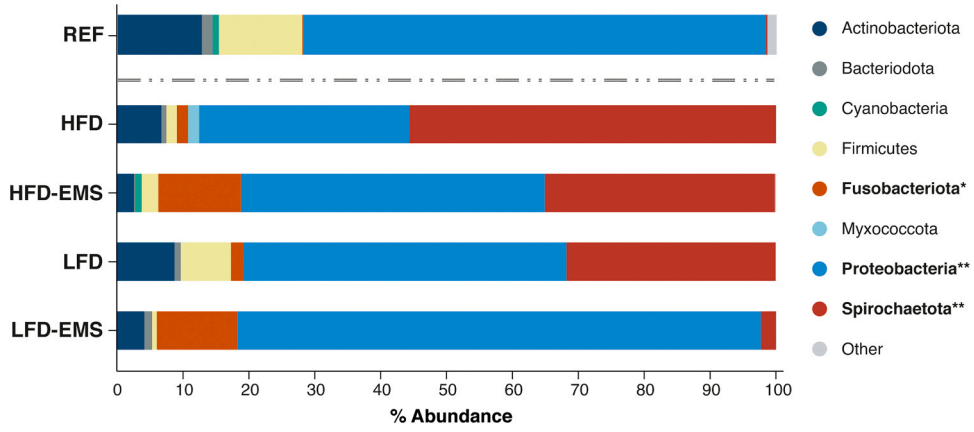

Fig. 2. Stacked bar chart representing the relative abundance of bacterial phyla for each of the experimental diets (HFD, HFD-EMS, LFD, LFD-EMS) compared with the reference group (REF) of fish with the same genetic background not exposed to extreme heat temperatures. Superscript asterisks in different phylum indicate significant differences (Two-way ANOVA, P < 0.05) due to emulsifier supplementation (*) or both emulsifier supplementation and lipid level reduction (**).

varied from 55,6 % in HFD fish to 2.26 % in LFD-EMS fish with the addition of emulsifier and the reduction of dietary lipid level.

3.3. Water microbiota composition

Nanopore sequencing of 12 water samples produced a total of 2186,454 reads (~180,000 reads per sample) with a N50 length of 1.56 kb (Fig. S4a). Rarefaction curves for all the samples were approaching horizontal asymptote, suggesting adequate sequencing depth (Fig. S4b). Comparison of richness (Chao1 and ACE) and alpha diversity (Shannon and Simpson) estimators did not show significant differences between groups (Fig. 5a-d). Microbial composition at phylum level in all the groups was characterized by the dominance of Proteobacteria (37.5–47.8 %), Bacteroidota (16.2–26.5 %) and Cyanobacteria (14.5–20.1 %) phyla (Fig. 5e). However, no statistical differences (Kruskal-Wallis test) were found in these three phyla or in the remaining phyla above 1 % abundance. In the same line, no differences were found in lower taxonomic levels regarding beta diversity analysis. Focusing on the highly abundant *Brevinema* genus of gut samples, sequences of this taxa were detected in water samples but only at residual levels (0.018–0.072 %).

3.4. Bayesian network and functional profiles of gut microbiota

To build the BN, a SAMBA filter was applied to the raw data to get rid of the bacterial taxa when normalized counts were close to zero. The initial model was composed by a total of 98 nodes, which cover 94.24 % of the total average abundances of the microbial population (data not shown). After filtering by Leiden hierarchical clustering, we identified up to eight clusters directly connected with the two experimental variables (Emulsifier & Lipid level) (Fig. 6). This interconnected network was composed by 65 nodes (89.2 % abundance), including 17 out of 19 taxa that drive the separation between experimental groups by PLS-DA. With the objective of comprehending the functional profile of these modelled bacterial associations, an inferred metagenome pathway analysis was performed, and intriguingly two out of eight clusters (Cluster 1, 3) exhibited a significant functional enrichment (Fig. 7). Cluster 1, with a significant enrichment in the pathways related to *Vibrio cholerae* infection and Biosynthesis of various other secondary metabolites, was specially represented by *Thauera* and *Vibrio* with a higher

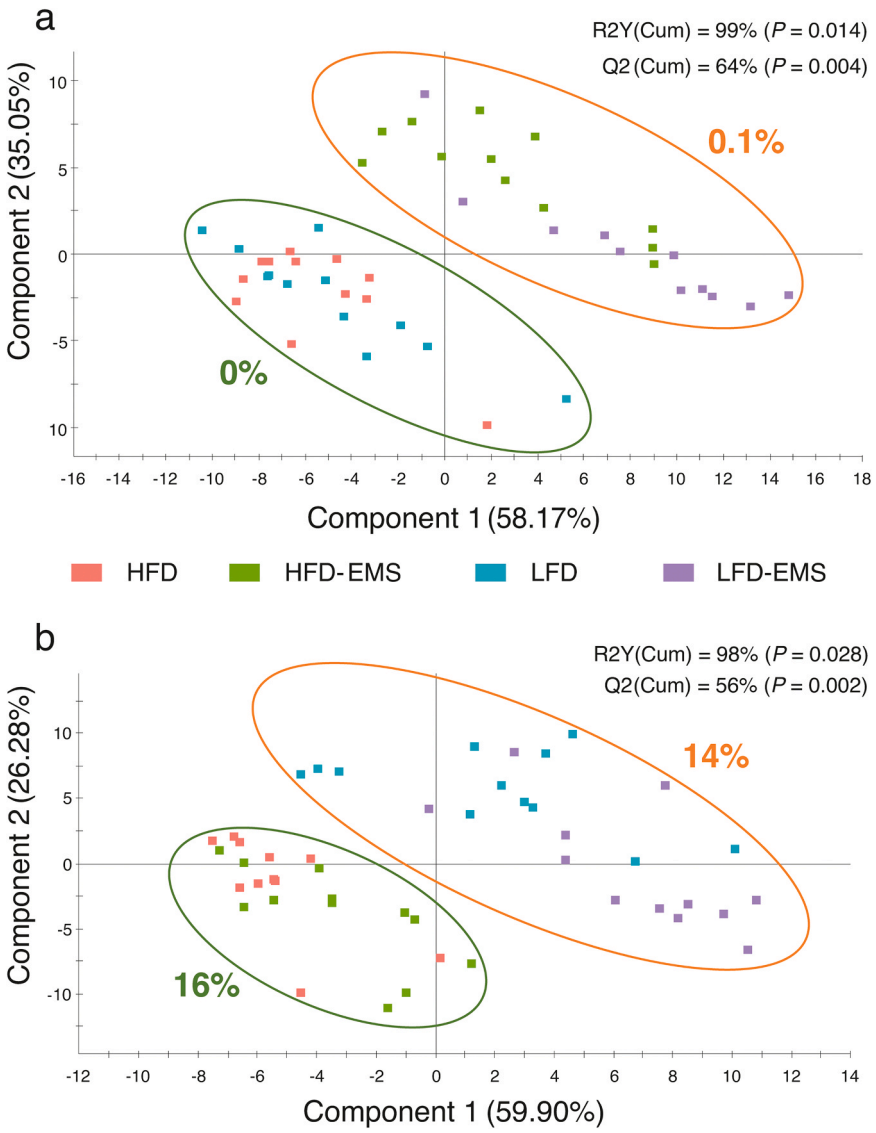

Fig. 3. Two-dimensional representation of the distribution of samples (n = 12) between the two first components of partial least squares discriminant analysis (PLS-DA) model driving the separation of groups based on emulsifier addition (a) and lipid level (b).

abundance (> 0.5 %) and discriminant value in the PLS-DA. Cluster 3 is globally more influenced by the variable Emulsifier, resulting in the enrichment of four additional metabolic routes (six in total): Biosynthesis of type II polyketide products, Bacterial invasion of epithelial cells, Isoflavonoid biosynthesis and Staurosporine biosynthesis. This functional enrichement was commanded by five abundant and discriminant bacteria (*Streptomyces*, *Blastococcus*, *Clostridium*, *Pseudomonas* and *Brevinema)*. Both *Pseudomonas* and *Brevinema* emerged as the two most important nodes as they play a pivotal role as parent and, thereby, controlling the abundance of other bacteria. In addition to that, *Brevinema* was not only highly connected within the cluster, but also with nodes belonging to other clusters via *Rothia* (Cluster 7) and Beijerinckiaceae (Cluster 5).

3.5. Blood stress markers

Plasma cortisol and glucose levels were altered by both dietary lipids and emulsifier supplementation (Fig. 8a-b). Thus, a statistically significant decrease of these two blood stress markers was found in HFD fish with the addition of the emulsifier, being the achieved values similar to those found in fish fed the LFD diet. Plasma levels of cortisol and glucose also decreased with the addition of the emulsifier to LFD, but this effect was not statistically significant. Of note, the abundance of the *Brevinema* genus in gut samples confirm the trend in plasma cortisol/glucose levels, but also made clearer and significant the effect of emulsifier supplementation in LFD (Fig. 8c).

4. Discussion

Exposure to high temperatures enhances basal metabolic activity and accelerates growth in farmed fish, but exceeding the upper thermal range tolerance triggers adverse physiological responses (Benítez-Dorta et al., 2017; Almroth et al., 2015; Islam et al., 2022; Balbuena-Pecino et al., 2019), modifying the use and reallocation of metabolic fuels (Alfonso et al., 2021; Volkoff and Rønnestad, 2020). In this line, we focused herein on dietary energy level and nutritional emulsifiers as a possible solution to mitigate heat stress in farmed gilthead sea bream. In fact, although several differences between endotherms and ectotherms exist in the thermal stress response, such as those linked to the dissipation of heat through the skin and tissular thermogenesis (Seebacher, 2009; Tattersall et al., 2012), the redistribution and storage of the main energy molecules, which particularly affect lipid homeostasis, also

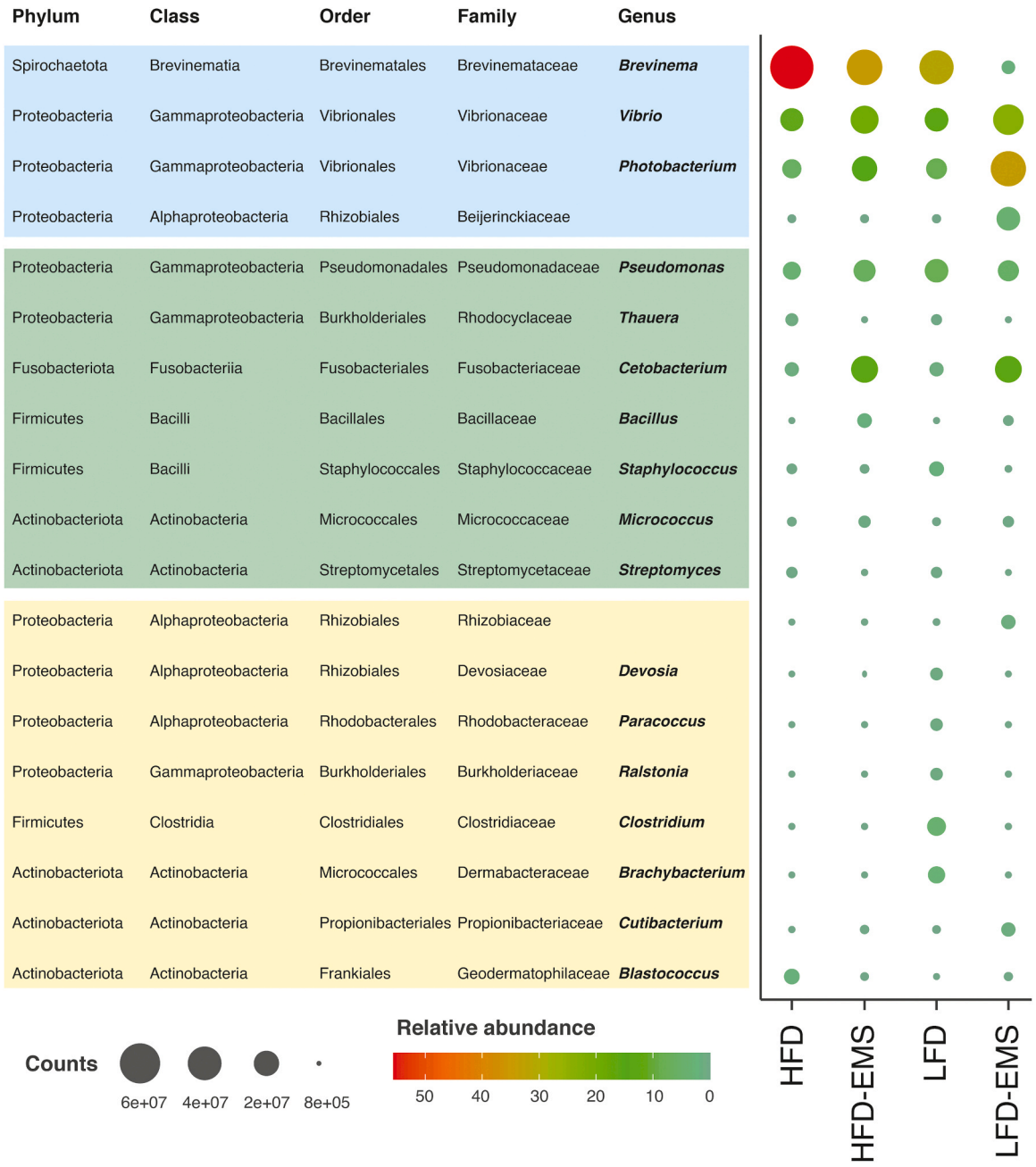

Fig. 4. Dotplot representing discriminant taxa (VIP > 1) with more than 0.5 % of relative abundance in at least one dietary group. Taxa are labelled depending if they are driving the separation by emulsifier addition (green; Fig. 3a), dietary lipid level (yellow; Fig. 3b) or both (blue). Colour scale represents the mean relative abundance, in percentage, of each taxon within each group. Size of the dots represents normalized counts in each group.

represents a central theme in warm-blooded animals. As described by numerous authors, in poultry, pigs, rodents and cows increasing hepatic lipogenesis and body fatness represent a part of the adaptive metabolic features that limit heat and ROS production, and thereby the risk of oxidative stress (Sanz Fernandez et al., 2015; Heng et al., 2019; Emami et al., 2021; Yasoob et al., 2022; Skibiel, 2024). Regarding this phenomenon, in fish, recent studies have also reported evidence that clearly link hepatic lipogenesis with changes in gut microbiota (Naya-Català et al., 2021a; Schoeler et al., 2023), and accordingly, the present study provides new insights supporting the reshape of gut microbiota as a subrogate marker of heat stress in fish, and gilthead sea bream in particular. Certainly, it must be noted that the bulk of available literature describing the intestinal microbiota of gilthead sea bream agrees on a general pattern with a dominance of the phylum Proteobacteria, followed by Firmicutes, Actinobacteriota and Bacteroidota (Estruch et al., 2015; Firmino et al., 2021; Moroni et al., 2021; Naya-Català et al., 2021b; Piazzon et al., 2020). Accordingly, the same phylum-level distribution was found herein in fish not subjected to episodes of extreme temperatures (REF fish). However, major shifts occurred in the gut microbiota of fish exposed to extreme temperatures with the displacement of the typically dominant Proteobacteria by the Spirochaetota phylum. This latter is commonly associated to marine environments, but its presence generally represents only a small fraction of the gut microbiota population (Naya-Català et al., 2022; Rimoldi et al., 2020). Therefore, a substantial shift in the microbiota suggests a potential risk to the homeostasis and stability of the entire community, as the loss or reduction of important bacterial taxa, can in fact give rise to invasions of potential pathogens and the creation of a dysbiotic environment

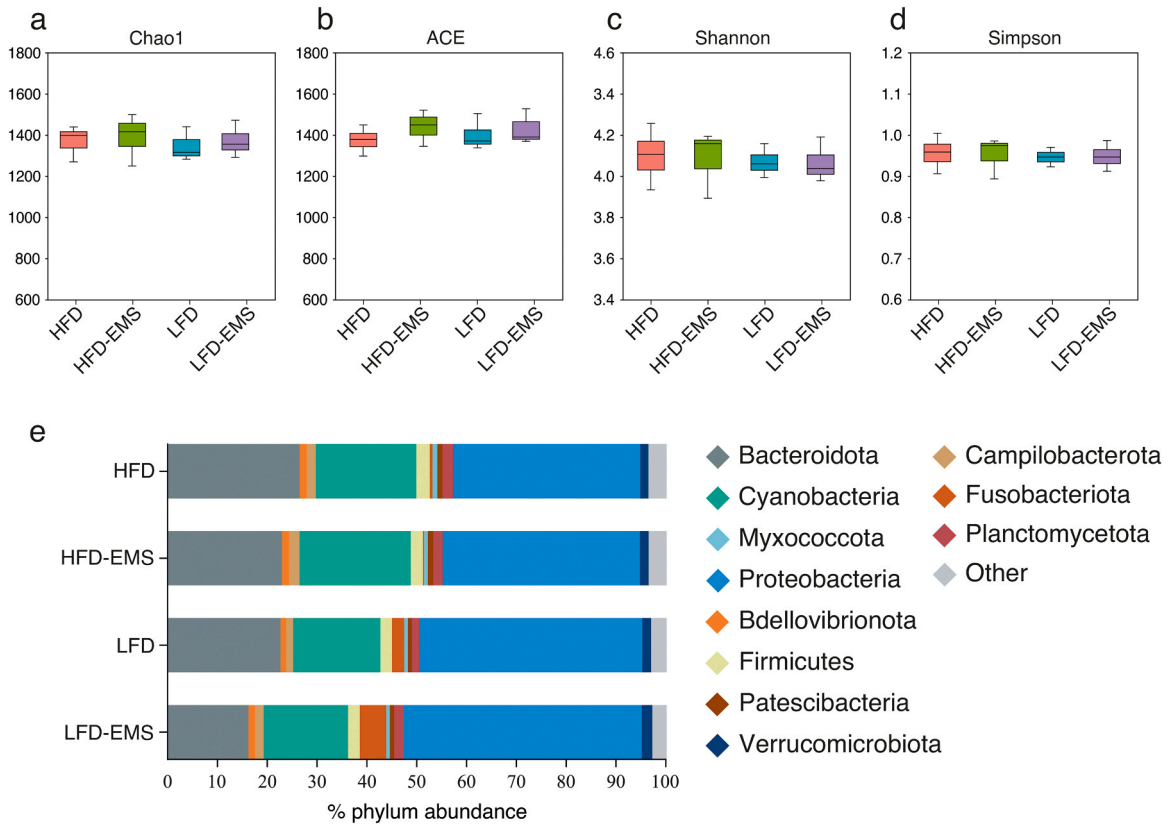

Fig. 5. Boxplots representing (a,b) richness estimators (Chao1 and ACE) and (c,d) diversity indexes (Shannon and Simpson) of the bacterial microbiota of rearing water. (e) Barplots representing bacterial distribution at phylum level of water samples.

(Infante-Villamil et al., 2021).

The role of diet as a key factor in modulating the intestinal microbiota was also evidenced at phylum-level in the present study, where the different experimental diets lead the different regulation of the intestinal microbiota following the achievement of historical water temperatures at our latitude during the extreme hot summer 2022 (Fig. 2). The most notable changes occurred in the HFD group, where the Spirochaetota phylum accounted for over 50 % of the total microbial abundance. However, this proportion was reversed with both the decrease of the dietary lipid level and the addition of the emulsifier, which would allow to lower the energy investment of fish in the digestion process and/or heat production with an excess of energy metabolic fuels, alleviating the negative effects of thermal stress as stated before in Nile tilapia (*Oreochromis niloticus*) (Wangkahart et al., 2022). Indeed, the independent addition of these two factors in the diet (HFD-EMS and LFD groups) shaped similar microbiota profiles, while the combination of both variables (LFD-EMS) shaped the lowest abundances of Spirochaetota phylum, suggesting an additive effect. This trend is also confirmed by the alpha diversity analysis, which revealed a significant lower biodiversity of the HFD group in comparison to LFD-EMS that also exhibited higher values of Chao1 and ACE indices and almost a complete reversion of the microbiota phyla towards the values achieved within the normal temperature range (Figs. 1 and 2). These findings agree with the observations made by Sánchez-Cueto et al. (2023) and Zhou et al. (2022), which also described a reduction in the bacterial biodiversity as a direct consequence of a thermal stress in greater amberjack's (*Seriola dumerili*) and rainbow trout (*Oncorhynchus mykiss*), respectively. Although the effect of environmental temperature on the alpha diversity are not entirely consistent in the scientific literature, an overall reduction of the population complexity and variability could be associated to an increasing chance of developing dysbiosis due to a microbiota disequilibrium (Kriss et al., 2018; Sánchez-Cueto et al., 2023).

In the present study, the nutritionally mediated effects on the composition of gut microbiota populations were even exacerbated at lower taxonomic levels (Fig. 3). Indeed, both dietary lipid levels and the presence of emulsifier showed a clear discriminant role, which allowed to identify several abundant genera with a different dependence on the basis of their relationship with the experimental variables. According to this, we have established three main taxonomic groups (Fig. 4), which primarily reflect the presence of the emulsifier in the case of *Pseudomonas, Thauera* and *Cetobacterium* cluster, while other bacterial taxa including *Ralstonia, Clostriduium* and *Brachybacterium* were apparently more responsive to the dietary lipid level. Finally, a third group was composed by highly abundant taxa (*Brevinema*, *Vibrio, Photobacterium*, and *Beijerinckiaceae* family), being their abundance shaped by both the emulsifier and the dietary lipid level. This cluster contains important members of the gilthead sea bream gut core microbiota under an opposite effect of the experimental conditions. Indeed, both *Photobacterium* and *Vibrio* are typically found in non-stressed gilthead sea bream (Ruiz et al., 2024). Our findings suggest that dietary intervention involving reduced fat content and the addition of emulsifiers reverted intestinal conditions, defining an LFD-EMS enterotype closer to a non-heat-stressed microbiota profile, which included Vibrionaceae taxa. Comparing gilthead sea bream results with previous studies in grass carp (*Ctenopharyngodon idella*; Zhou et al., 2018), rainbow trout (Zhou et al., 2022) or yellowtail (*Seriola lalandi*; Soriano et al., 2018), it is difficult to establish a common pattern linking changes in gut microbiota composition with thermal stress, dietary lipid level or nutritional emulsifiers. The discrepancy on the achieved results also applies to intra-species comparisons, as evidenced by the recent study of Ruiz et al. (2023a), who reported in gilthead sea bream a high abundance of the genus *Brevundimonas* in concurrence with a significant reduction of *Acinetobacter*, *Corynebacterium* and *Peptoniphilus* with the use of bile salt supplemented diets. This apparent lack of uniformity can be attributed to a

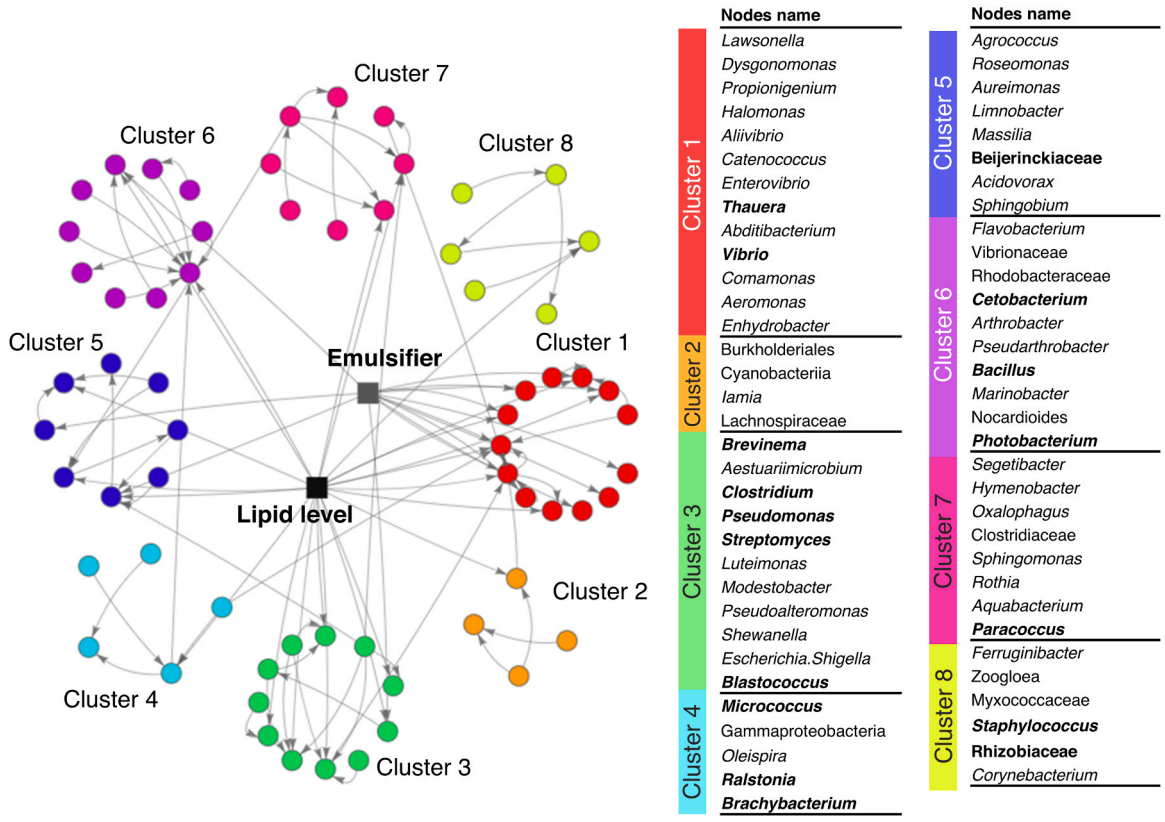

Fig. 6. Bayesian Network obtained with SAMBA. Circles represent bacterial taxa and squares represent experimental variables (Emulsifier; Lipid level). Each colour is specific to a bacterial cluster (1–8). The complete list of bacterial taxa composing the BN are reported in the table, divided by clusters. In the table, taxa listed in the Fig. 4 Dotplot are marked in bold.

a

	Pathway description	pvalue	p.adjust
Cluster 1	*Vibrio cholerae* infection	<0.0001	0.0005
	Biosynthesis of various other secondary metabolites	0.0059	0.039
Cluster 3	*Vibrio cholerae* infection	0.0004	0.0104
	Staurosporine biosynthesis	0.0005	0.0104
	Biosynthesis of type II polyketide products	0.0006	0.0104
	Biosynthesis of various other secondary metabolites	0.0025	0.023
	Bacterial invasion of epithelial cells	0.0026	0.023
	Isoflavonoid biosynthesis	0.003	0.023

b

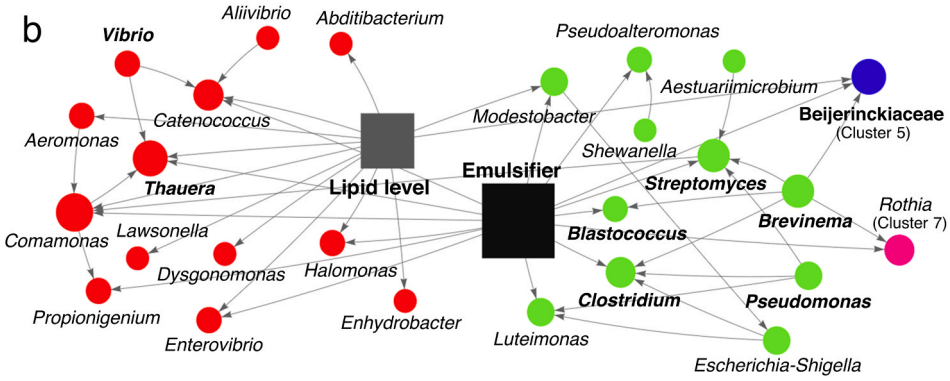

Fig. 7. (a) Table reporting the two Clusters (1; 3) with significant (p-adjusted < 0.05) functional enrichment. (b) A detailed extract of the two Clusters taken from the total BN. Red circles are bacterial taxa belonging to Cluster 1, green circles to cluster 3, blue and pink to cluster 5 and 7, respectively. Squares represent experimental variables (Emulsifier; Lipid level).

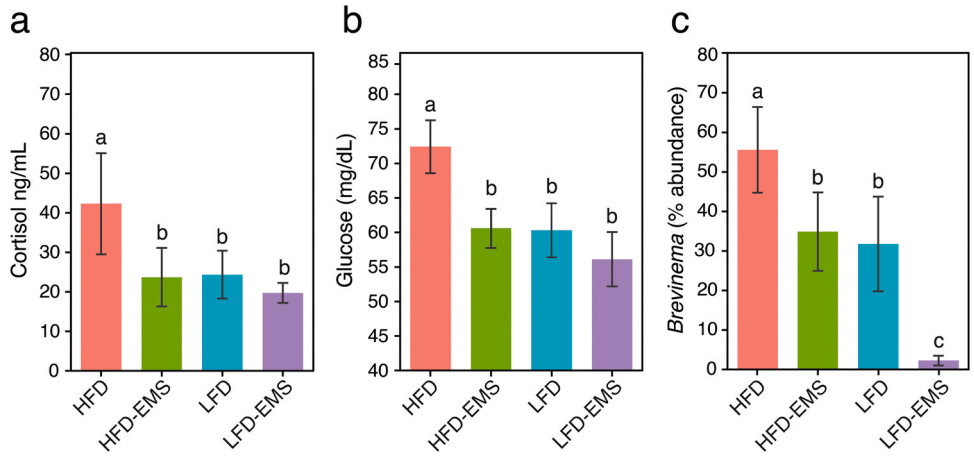

Fig. 8. Blood biochemistry analysis of cortisol (a), glucose (b) and *Brevinema* genus abundances in anterior intestine microbiota (c) of the four experimental groups (n = 12). Significant differences (one-way ANOVA, Dunn's post-test for blood stress indicators and Kruskal-Wallis test for relative abundances) are represented by superscript letters (*P < 0.05).

different fish strain, life background, developmental stage or culture rearing system among other factors, which makes difficult (if not impossible) the comparisons of gut microbiota results within and between farmed/wild fish species. In particular, in gilthead sea bream, this is supported by recent studies showing how the gut microbiota composition is modulated by age, sex, season, diet, rearing density and host genetics (Piazzon et al., 2019, 2020; Naya-Català et al., 2022; Toxqui-Rodríguez et al., 2024). This, together with the great microbial diversity at low taxonomic levels, makes necessary the identification of bacterial biomarkers in close association with a given experimental condition. Therefore, in the absence of a single gold standard for fish intestinal microbiota, there is an urgent need to expand the list of potential microbiota biomarkers not only for their taxonomic identification, but above all for their functional role in interaction with the host (He et al., 2021).

At a closer look, it must be noted that *Brevinema* genus appeared in our experimental model as a highly abundant bacteria taxa that becomes highly influenced by the temperature and nutritional condition. In fact, *Brevinema* is a microaerophilic and gram-negative motile genus with an optimum growth temperature range between 30ºC and 34ºC that has been detected in the intestine of a number of fish species, including European sea bass (*Dicentrarchus labrax*; Alfonso et al., 2023), chinook salmon (*Onchorhynchus tshawytscha*; Steiner et al., 2022), rainbow trout (Brown et al., 2019), tilapia (*Oreochromis spp.*; Paimeeka et al., 2024), white cachama (*Piaractus brachypomus*; Castañeda-Monsalve et al., 2019), and gilthead sea bream (Huyben et al., 2020; Naya-Català et al., 2021a; Piazzon et al., 2019; Quero et al., 2023). Although a wide range of bacterial groups belonging to Spirochaetota phylum live in aquatic environments (Paster, 2010) the genus *Brevinema*, even in a gilthead sea bream context, has been found in association with host mucosas, remaining mostly absent in the surrounding water and sediments (Quero et al., 2023). This agrees with our results, even considering the actual differences between sequencing platforms as previous studies demonstrated that amplicon-based bacterial compositional data is not fully comparable (Toxqui-Rodriguez et al., 2023). However, *Brevinema*, and other genera belonging to phylum Spirochaetota, which were detected by both sequencing platforms, exhibited different patters between water and intestine. Hence, the only residual levels identified in water samples confirmed that the great changes observed in the gut microbiome are not mirrored in the compositional data of water samples, suggesting that the association with heat-stress episodes was not driven by environmental colonization but is specifically associated with the host.

In any case, gilthead sea bream studies, analysing the temporal succession of the intestinal microbiota, highlighted a pronounced increase of *Brevinema* with advancing age (Piazzon et al., 2019), and through the production cycle from residual levels in winter (< 0.001 %) to 4 % in the warm season (Naya-Català et al., 2022). Likewise, in other farmed fish such as chinook salmon, it has been described the gradual increase of *Brevinema* abundance in both faeces (transient microbiota) and mucosal samples (adherent microbiota) with the temperature rise from 8ºC to 20ºC in a recirculating aquaculture system (Steiner et al., 2022). Furthermore, also in gilthead sea bream, a previous study highlighted a strong positive association between the hepatic expression of key lipogenic *scd1* gene and the presence in the intestine of *Serratia*, but also *Brevinema* (Naya-Català et al., 2021a).

Altogether, the above findings support the idea that *Brevinema* possesses a metabolic capacity that allows it to grow significantly in abundance due to thermal stress, exploiting and negatively enhancing a condition of imbalance in intestinal homeostasis. The construction of a Bayesian Network model by means of the SAMBA platform has in fact emphasized a multi-connected *Brevinema* that takes a leading role within its cluster, connecting and influencing some other bacteria such as *Clostridium, Streptomyces, Blastococcus, Rothia* and Beijerinckiaceae family (Figs. 6–7). The sum of these connections depicts a potential dysbiotic risk that becomes evident by functional enrichment analysis, as it includes pathways correlated with *Vibrio cholerae* infections, bacterial invasion of epithelial cells and biosynthesis of biological active and toxic molecules such as staurosporines and polyketides. In this line, potential unfavourable conditions associated with a higher abundance of this genus have also been connected in the red hybrid tilapia with infectious diseases (Paimeeka et al., 2024). Likewise, in salmonids, the rise of *Brevinema* has been associated to the administration of chemotherapeutants (oxytetracycline) (Payne et al., 2022), also linked to an increased susceptibility to infectious disease and the up-regulated expression of immune relevant genes (Brown et al., 2019; Li et al., 2021). However, as already pointed out before, our dietary intervention was able to reverse, at least in part, the enterotype phenotype associated with heat episodes, resulting in a decreased *Brevinema* abundance in favour of other bacterial taxa belonging to Proteobacteria phylum, such as *Vibrio, Photobacterium* and family Beijerinckiaceae. Remarkably, the changing *Brevinema* abundance was also closely associated with changes in conventional blood-stress markers (e.g. cortisol, glucose), according to which the intestinal *Brevinema* mimicked the changing plasma cortisol and glucose levels, being achieved the lowest values with the combination or low dietary lipid levels and emulsifier supplementation in fish fed the LFD-EMS diet.

In summary, extreme heat episodes disrupted the homeostatic relationship between the gut microorganisms and the host, resulting in a disproportionate amount of *Brevinema* taxa in the intestine of farmed gilthead sea bream. Revisiting the current literature, the increased

abundance of this opportunistic microorganism is becoming a generic marker of heat stress in farmed fish, and gilthead sea bream in particular. However, further research is needed to clarify and delve deeper into the mechanisms that constitute the basis of the Brevinema-host response against the global warming across species, developmental stages and rearing systems. In that sense, the construction of a BN model has contributed to disentangle the complex association of Brevinema with other bacteria taxa, making sense the functional enrichment analysis to the close association of *Brevinema* with the nutritionally mediated changes in conventional blood-stress markers. Altogether, these results open the door to monitor and delineate the best nutritional and environmental strategies to mitigate the negative impact of global warming in aquaculture production.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. S. Cools reports a relationship with Nukamel NV that includes: employment. E. Croes reports a relationship with Nukamel NV that includes: employment. H. Boon reports a relationship with Aquaculture Experience that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102566.

Data availability

Data will be made available on request.

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