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Abstract

The effect of toxin-producing cyanobacterial blooms on fishes health has been investigated extensively, but no study to date has evaluated this effect on fish-associated microbiota. In this study, we test the effect of pure microcystins and of crude extracts of metabolites from *Microcystis aeruginosa* cultures on the composition of gut bacterial microbiota in Medaka fishes (*Oryzias latipes*) exposed for 28 days in a microcosm experiment. A 16S rRNA-based marker gene-based approach was used to investigate the composition of bacterial communities. Results show that fish gut community compositions differ from those occurring in the water, and among individual fishes. Exposure to extracts, rather than pure microcystin, has a significant influence on gut community composition, with a marked increase in relative abundances of pathogen-related bacteria (genera *Nocardia* and *Mycobacterium*) in the presence of one extract, and of bacterial orders Sphingomonadales and Saprospirales in the other. We suggest that compounds identified in the cyanobacterial extracts, but not microcystin LR alone, alter the composition of bacterial communities, with possible consequences for various biological functions in fishes. This pioneer microcosm experiment indicates that cyanobacterial blooms probably have an effect on fish gut microbiota and associated functions, including toxin degradation and feed efficiency, and should be further explored.

Introduction

Cyanobacterial proliferations occur worldwide in freshwater, estuarine and marine environments, when environmental conditions allow (*i.e.* nutrient inputs, light intensity, high temperatures, pH and CO_2 concentration). In recent years, massive cyanobacterial blooms increased in frequency and persistence around the world, causing serious threats to aquatic ecosystems[1,2]. Indeed, cyanobacteria produce numerous bio-active secondary metabolites, including various cyanotoxins, that remain and bioaccumulate into the environment, especially after bloom senescence[3–5]. The most widespread cyanotoxin is microcystin-leucine-arginine (MC-LR) which can accumulate in the liver of aquatic vertebrates including fish[6,7]. Microcystins are produced, among others, by *Microcystis*, the most common cyanotoxin-producing and bloom-forming genus in freshwater ecosystems[8].

Over the last decade, MC-LR effects upon fish ecotoxicology have been documented in some species[9–12]. Among these, the Medaka fish (*Oryzias latipes*) is a model of choice for toxic effects investigation thanks to its resistance to stress and diseases. Natural cyanobacterial blooms, lab cultures, biomass extracts and pure microcystins (*e.g.* MC-LR) were shown to adversely impact its development, reproduction, and to induce specific organ alterations, notably hepatotoxicity and cardiotoxicity[7,13–15]. However, despite the documented impact of cyanobacterial blooms on fish health, no study has to our knowledge investigated the link between these events and the composition of fish microbiota.

In ecology, the holobiont, *i.e.* the super-organism composed by one pluricellular host and its associated microorganisms constituting its microbiota, is increasingly acknowledged as a relevant level of investigation[16]. Indeed, microbiota play multiple fundamental roles in host physiology, including in nutrition, immunity, protection and behavior[17]. In the context of aquaculture and fisheries, many investigations have recently highlighted the importance of fish microbiota for feed efficiency, and pathogen resistance[18]. Located at the interface between an organism and its environment, the microbiota is exposed to the latter, and should thus be accounted for in ecotoxicology studies. In this perspective, some cyanotoxins, known for their antimicrobial effect, could directly impact the composition of fish microbiota. Such effects have already been shown on the gut microbiota of the branchiopod *Daphnia,* for instance, resulting in alteration of their tolerance to toxic cyanobacteria[19]. Cyanobacterial secondary metabolites could also represent nutrient sources that select gut microbes able to use them.

The aim of the present study is to test whether changes occur in fish microbiota compositions when hosts are exposed to cyanobacteria in a microcosm setup. As a natural cyanobacterial

bloom is difficult to stimulate in the laboratory, we investigated the effects of three cyanobacterial extracts (two from distinct microcystin-producing *Microcystis aeruginosa* strains and one pure MC-LR extract) on water-borne bacterial populations and on the Medakas' gut microbiome composition. Cyanotoxins concentrations were chosen to mimic a low-level intoxication, just above the norm for drinking water[20] to see how chronic exposure may impact gut bacteria. Bacterial communities are investigated using an Illumina-based metabarcoding approach on the V4-V5 region of the 16S rRNA-encoding gene. Potential consequences for nutrition and pathogen resistance are discussed.

Materials and methods

Secondary metabolites in extracts of Microcystis aeruginosa *strains*

Three different metabolite mixtures were used in exposure experiments. The first consisted in pure microcystin-LR resuspended in 50% ethanol/water solution and then partially evaporated (Novakit, France). The other two were extracted from cultures of *Microcystis aeruginosa* strains Paris Museum Collection (PMC) 728.11 (Extract 1) and Pasteur Culture Collection (PCC) 7820 (Extract 2) available upon request from the National Museum of Natural History and Institut Pasteur collections, respectively. Both were cultured at 25°C in Z8 media (Rippka 1988, 16h: 8h light/dark cycle at 60 $\mu mol.m^{-2}.s^{-1}$). Cell cultures were sonicated in methanol, centrifuged (10 min, 4°C, 3,500 g), methanol was evaporated, extracts were resuspended (EtOH 50%), and ethanol was evaporated. Microcystin concentration was measured as MC-LR equivalents (MC-LR eq.) using the Novakit Microcystin-ADDA ELISA kit (France). Metabolites composition of the extracts were analyzed on an UHPLC (Ultimate 3000, ThermoFisher Scientific) coupled with a high-resolution mass spectrometer (ESI-Qq-TOF Maxis II ETD, Bruker). The analyte annotations were performed according to precise mass, isotopic and fragmentation MS/MS patterns, as previously described[14].

Exposure experiments and monitoring

Adult male Medaka fishes were used in all experiments. Four treatments were applied: a solvent control without any cyanobacterial metabolite; exposure to Extract 1 (~2.5 µg MC-LR eq.L^{-1}); exposure to Extract 2 (~2.5 µg MC-LR eq.L^{-1}); and exposure to pure microcystin (~2.5 µg MC-LR eq.L^{-1}). Each of the four treatments was applied on three replicate 10-liter aquaria, each containing six fishes that were acclimatized 10 days prior to the beginning of the exposure. Fishes were then exposed for 28 days at 25°C±1°C. They were fed twice a day

with T-0.3 Nutra HP (Skretting, Netherlands). Every two days, the aquaria were cleaned, half of the water was replaced, and the extracts or MC-LR were renewed in totality in the concentrations described above. Water temperature, conductivity, pH, nitrate and nitrite were monitored every two days, and samples taken to further determine the total microcystin content using the ELISA kit.

Fish dissection and tissue fixation

At the end of the 28 days' exposure, three fishes (n=3) were sampled from each of the 12 aquaria, except for aquaria E (four fishes), F and I (two fishes each, see Table S1). Fishes were anesthetized in 0.1% tricaine methane sulfonate (MS222; Sigma, St. Louis, MO), sacrificed, dissected, and the whole intestine (including content because of small size) was sampled and flash-frozen in liquid nitrogen. Aquarium water (50 mL) was filtered on a 0.22 µm nitrocellulose filter, then filters were flash frozen.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from the intestine of 2 to 4 (mostly 3) replicate fishes from each aquarium, from water-filters and from fish food using a ZymoBIOMICS™ DNA mini kit (Zymo Research, CA), following mechanical lysis (bead-beater, 6 minutes, maximum speed). A ~500 bp fragment of the rRNA-encoding gene corresponding to the V4-V5 variable region of *Escherichia coli* was amplified using 515F and 926R primers[21] and sequenced on an Illumina MiSeq platform (2 X 250 bp, paired-end sequencing, Genoscreen, France). Raw reads were deposited into the GENBANK Sequence Read Archive (SRA) database under accession number PRJNA517613 (samples SAMN10839113 to SAMN10839169, see table S2).

Sequence analysis

Raw reads were demultiplexed, quality checked and trimmed, and paired reads were assembled in contigs using FLASH[22]. Sequence analysis was performed using QIIME2[23]. Amplicon Sequence Variants (ASVs[24]) were identified using DEBLUR[25]. Chimeric sequences were identified using UCHIME (*de novo* chimera detection) and then removed[26]. Taxonomic affiliations were obtained by the sklearn-based classifier (GreenGenes 13-8-99 release). Sequences matching "Chloroplast" and "Mitochondria" were discarded. Rarefaction curves, alpha and beta diversity indices were generated using a sampling depth of 8,132 corresponding to the lowest number of quality-filtered reads obtained in a sample. Two gut

samples (one from Extract 1 and one from Extract 2 experiments) were outliers in terms of (very low) ASVs number compared to samples from the same conditions, and were discarded from further analysis. A guide phylogenetic tree was produced to compute UniFrac distances[27]. Principal coordinates analysis (PCoA) plots based on Weighted and Unweighted UniFrac distances (WU and UU) as well as Bray Curtis (BC) dissimilarities were generated using ggplot2[28]. Community richness were compared using ANOVA, and compositions were compared using PERMANOVA. A Venn diagram was drawn using the web-based software available at http://bioinformatics.psb.ugent.be/webtools/Venn/.

Results and Discussion

The analyzed parameters (temperature, conductivity, nitrites, pH and exposure levels measured as MC-LR equivalents) were stable within a given aquarium during the 28-days experiment (Table S1). Analysis of Extracts 1 and 2 from the *M. aeruginosa* strains revealed very different compositions in secondary metabolites besides MC-LR, present at various concentrations, including cyanopeptolins, aeruginosins, aerucyclamides, anabaenopeptins, microviridins and/or microginins (Figure S1). Exposure levels ranged between mean 1.1 ± 0.4 µg MC-LR eq.L^{-1} (Extract 1) and 5.6 ± 1.1 µg MC-LR eq.L^{-1} (Extract 2), with 2.3 ± 0.7 µg.L^{-1} in the pure MC-LR treatment. They were slightly above the norm for drinking water (1.0 µg eq. MC-LR eq.L^{-1}), and represent chronic exposure levels rather than the acute levels expected during or after a very intense bloom event[20]. These conditions were previously shown to lead to observable lesions on the liver of young and adult fish, but not to major dysfunction[14, 29-31]. The toxicity on hosts is thus limited, as are potential associated indirect effects on microbiota linked for example to organ malfunction leading to changes in the physico-chemical environment of microorganisms. Therefore, we expect to rather observe the direct effect of the MC-LR and the extracts on the microbiota itself.

A total of 1,861,481 assembled paired-end reads were obtained from 12 water samples (3 replicates per treatment) and 33 gut samples (7 to 9 per treatment, Table S1), of which 1,063,328 from bacterial origin passed quality filters. Deconvolution resulted in a total of 662 ASVs. Rarefaction curves reached saturation for all samples, indicating that the dataset accurately represents the bacterial community (not shown). Depending on the sample, between 8,132 and 37,511 assembled sequences were obtained, representing 18 to 178 ASVs (Table S2). Comparisons indicate that the gut samples from specimens exposed to extracts 1 and 2 display overall higher diversity of ASVs and Shannon indices (Figure S2-3).

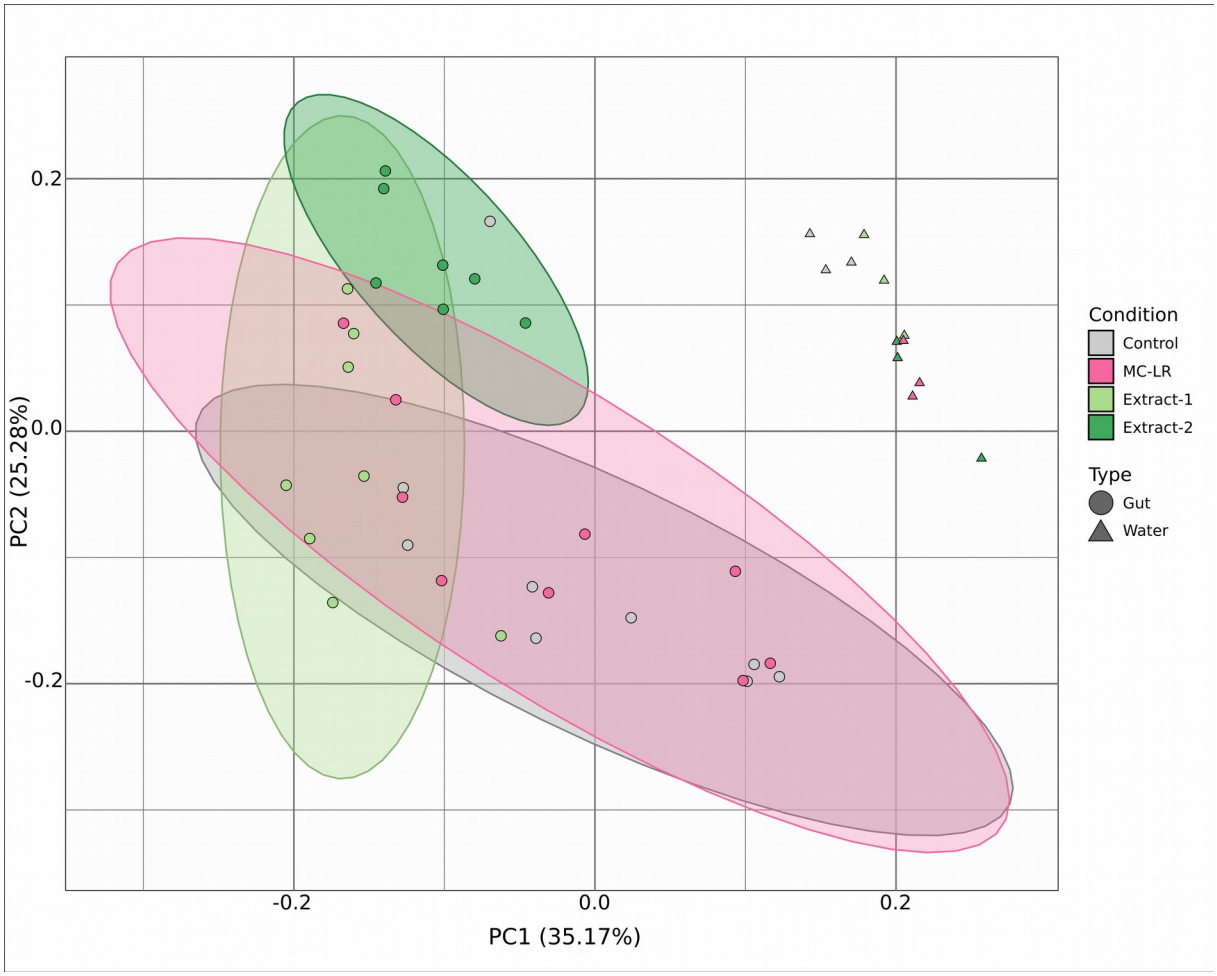

Figure 1. Principal Coordinates Analysis based on Weighted Unifrac dissimilarity metrics. Ellipses correspond to 95% confidence intervals for gut samples exposed to each of the four treatments.

Bacterial communities in the water are similar

Bacterial communities compositions were not significantly different among water samples whatever the treatment (WU distances, PERMANOVA $p>0.09$), and clustered together in the principal coordinates analysis (PCoA, Figure 1). Similar results were observed in PcoA plots obtained using UU and BC dissimilarities (figure S4). These communities were dominated by Burkholderiales (Betaproteobacteria, mean 60.1±13.5% of reads), Saprospirales (Bacteroidetes, 11.8±6.3%), Sphingomonodales (8.1±4.6%) and Rickettsiales (8.1±8.8%) (Alphaproteobacteria, Figure 2). Interestingly, abundances of Leptospirales (Spirochaetes), some of which are known to be animal pathogens, were significantly lower in the presence of cyanobacterial extracts or MC-LR compared to control (p-value<0.01), suggesting selective antibacterial effects of these compounds. In total, the water samples displayed 390 ASVs, of which 145 also occurred in gut samples.

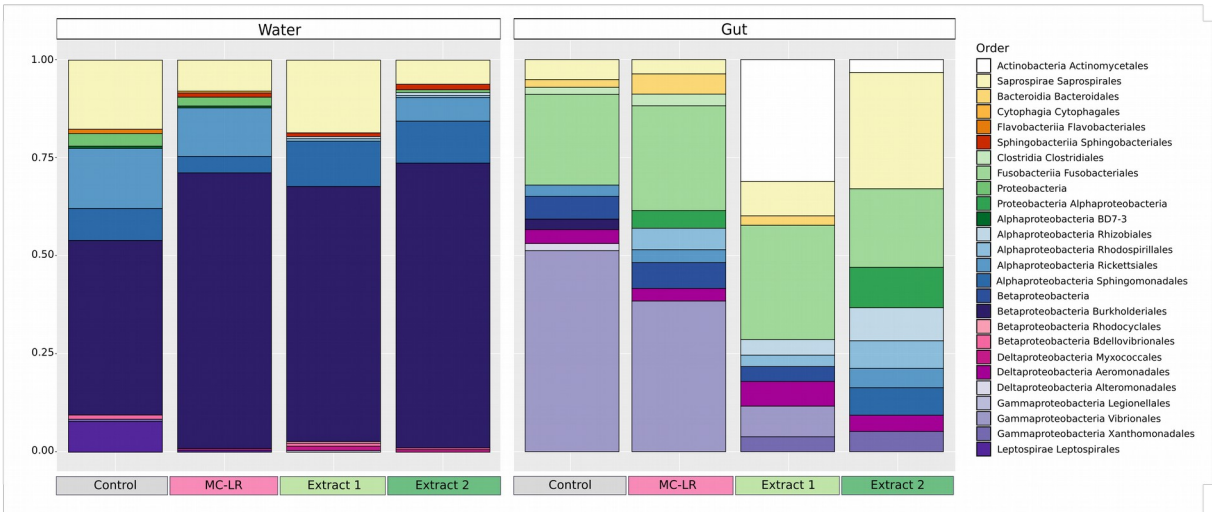

Figure 2. Relative abundances of major bacterial orders (mean values) present in water and gut samples in each treatment.

Gut bacterial community composition is influenced by treatment

Communities compositions from gut samples were different from those in the water (p-value<0.001) and well separated on the PCoA plots (Figure 1 and S4). Differences between fish gut and water communities have previously been reported in various studies[18,32–34]. In contrast to water, gut bacterial communities were much more spread on the PCoA plot (Figure 1), emphasizing high inter-individual variability within a given experimental condition. This variability is commonly observed among vertebrate hosts[34–37] and is not explained by the different aquaria used in this study (factor "aquarium" on gut community composition, p-value>0.4). Bacterial community compositions were not significantly different between control and MC-LR-exposed fish guts (p>0.7, Figure 1). These on the other hand strongly differed from compositions in guts of fish exposed to Extract 1 and Extract 2 (p-values<0.01), which also differed between them (p-value<0.01). The same results were obtained in UU and BC analyses (figure S4).

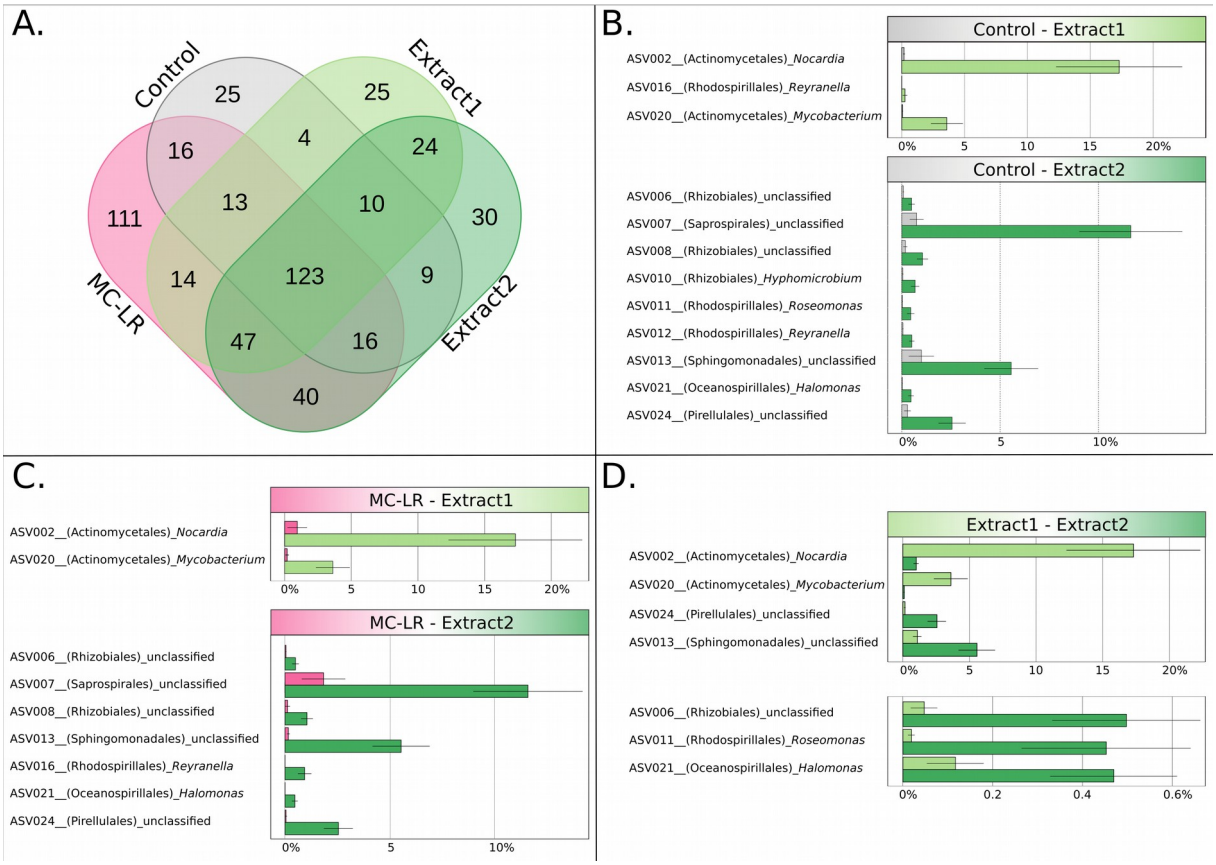

Figure 3. A. Venn Diagram displaying the number of gut bacterial ASVs shared among treatments. **B-D:** Major gut ASVs displaying significant differences in relative abundance between two conditions (pairwise t-tests with Bonferroni correction, p-values< 0.01). The bar plots display the mean proportion of ASV in gut samples from each of the two treatments, named by their ID and their affiliated genus, when available. Among gut ASVs, 26 displayed significantly different relative abundances between at least two treatments, of which only the 12 that represented at least 1% of reads in at least one gut sample are listed.

Identity of stable and variable taxa and possible link with treatments

Overall, 507 distinct ASVs occurred in gut samples, of which 123 were shared among all treatments and 25 to 111 were unique to one treatment (Figure 3A). The 20 most abundant ASVs represented between 53.7 and 97.8% of total reads in a given individual. In each bacterial order, a single or two ASVs represented most of the reads. Significant variations in taxa relative abundances were observed among treatments (summarized at the Class, Order, Family and Genus levels in figure S5, and for ASVs in figure 3). Despite variation among treatments, order Fusobacteriales (Fusobacteria), dominated by a single ASV affiliated to *Cetobacterium somerae* (ASV253), was abundant in fish gut microbiota in all conditions (mean reads per treatment between 12.9% and 26.2%, Figure 2). *C. somerae* notably produces B12 vitamins beneficial to various hosts and plays an essential role in healthy host physiology in several species[32,38-40]. Vibrionales (Gammaproteobacteria) represented by two main ASVs,

namely ASV220 and ASV378, was the second most abundant bacterial order in three treatments (mean reads per treatment between 7.9% and 46.3%), but was almost absent in guts from fishes exposed to Extract 2 (mean=0.17%, Figure 2, Table S3, Figure S5). This order includes commensal and animal pathogenic genera which are common in fish guts[36,39,40]. Both taxa, *C. somerae* and Vibrionales, have already been reported in congeneric *Oryzias melastigma* (marine Medaka), and in a small 16S rRNA clone library obtained from *O. latipes* gut[39,40].

Various other taxa were related to common fish pathogens, for example *Aeromonas salmonicida* (ASV237, mean 2.9 % of reads over all treatments[34]). Among these, two ASVs within Class Actinobacteria were significantly more abundant in guts exposed to *Microcystis aeruginosa* Extract 1 compared to Extract 2, Control and MC-LR treatments (Figure 3B-D, Figure S5). The first ASV was related to genus *Nocardia*, namely ASV002 (mean 17.2% of reads in Extract 1 samples, occuring in 7 of the 8 specimens), and the second to genus *Mycobacterium* (ASV020, 3.6% of reads in Extract 1 samples, present in 7 out of 8 specimens). *Nocardia* and *Mycobacterium* genera, both belonging to the family Mycobacteriaceae, are known as pathogens of animals, including fish[41]. Interestingly, Mycobacteria are frequently reported to be more abundant in guts of animals suffering inflammatory bowel diseases even though their role in these pathologies remains unclear[42,43]. Nevertheless, dysbioses (*i.e.* lasting changes of the microbiota), as those occuring in inflammatory bowel diseases, are known to favor the increase of bacterial pathogens abundances by disrupting the protective barrier provided by the healthy gut microbiota[44, 45]. Therefore, the change in bacterial community and the increase of Mycobacteria abundances may reflect a dysbosis induced by chronic exposure to Extract 1 (Figure S5).

In Extract 2 treatment, two other bacterial orders displayed significantly higher abundances compared to other treatments, namely Saprospirales (ASV007, 11.6% of reads in Extract 2 samples) and Sphingomonadales (ASV013, 5.5% of reads, Figures 2, 3B and 3C), and ASVs were present in all specimens. These orders are not reported to include animal pathogens, but some are able to degrade a wide variety of metabolites including complex carbohydrates, proteins and aromatic compounds[46]. For instance, plant-based diets promote Saprospirales population in fish guts[47]. Growth of Sphingomonadales members has been shown to correlate with that of cyanobacteria including *Microcystis*, and the former were suspected to benefit from organic matter produced by cyanobacteria[48,49]. Furthermore, several members of Sphingomonadales can use microcystins as nutrient sources[48]. Overall, observed increase of Saprospirales and Sphingomonadales abundances thus suggests that Extract 2

exposure selected microbiota members able to metabolize cyanobacterial secondary metabolites. By modulating gut microbiota compositions, relatively low cyanobacterial metabolites abundances could thus have serious effects on host feed efficiency.

Overall, results from this study first show that MC-LR alone had no noticeable effect on the composition of gut-associated bacterial communities as recently shown on pooled individuals of zebrafish[50], at the levels and duration applied here, while full extracts of *Microcystis aeruginosa* both had a significant effect. Full extracts contained various secondary metabolites besides MCs, among which some probably directly affect animal-associated microbial communities, either alone or together. For instance, aerucyclamides, some of which were identified here (Figure S1), are cyclic peptides that may present antimicrobial and cytotoxic activities. Cyanobacterial secondary metabolites may also include compounds interfering with bacterial quorum sensing, or exhibiting allelopathic or antibacterial properties[51]. These compounds may lead to dysbioses and their consequences, such as the increase in abundances of potential pathogens observed in Extract 1, or the increase in bacterial taxa that may use cyanobacterial metabolites as hypothesized in Extract 2. Possible functional consequences for fish health need to be further explored, but these changes in microbiota composition could alter feed efficiency, pathogen susceptibility or protective functions of the gut. Experiments from this study mimic what may happen in a lake after the end of a bloom, when compounds are released in the water following cyanobacteria senescence. Given the reported consequences of blooms for fish health in the wild and in aquaculture settings where they may greatly affect yields, this topic appears as a promising line of research in animal-bacteria interactions and a microcosm-based approach using extracts is an appropriate first step to tackle the issue. The next step will be to investigate the effect of direct exposure to cyanobacterial blooms, ideally in mesocosm setups, to test whether the actual presence of bacteria modifies these effects.

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Supporting Information Available

Supporting information contains additional data and analyses on the composition of cyanobacterial extracts, alpha diversity (observed ASVs and Shannon indices), beta diversity (PcoA plots and tests based on UU and BC metrics), and community composition at the Class, Order, Family and Genus levels. Three supporting tables are provided that summarize water parameters, samples ID and characteristics, and relative abundances, taxonomic affiliation and sequences of all ASVs. This information is available free of charge on the ACS Publications website.

Authors contribution

SD and BM conceived the study

MH, CD, BM and SD conducted the exposure experiments

SD, SH, AG, BM and MH performed molecular analyses, and analyzed the data

HH performed the analyses on liver.

SD, SH and AG drafted the manuscript, all authors contributed and agreed on the contents

Ethics statement

Experiments on fishes were conducted according to good practice, validated by comité Cuvier (Author. APAFiS#19316-2019032913284201) and under the supervision of accredited personnel (CD, BM).

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