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The impact of the anti-diabetic drug metformin on the intestinal microbiome of larval brown trout (*Salmo trutta* f. *fario*)

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Abstract

Background: The anti-diabetic pharmaceutical metformin is frequently analysed in the aquatic environment. Its impact on the fish microbiome is studied to get a deeper knowledge about the consequence of the metformin presence in river systems. Gut microbiome analyses were performed on larval brown trout with metformin including environmental concentrations. Therefore, the fish were exposed to metformin in concentrations of 0, 1, 10, 100, and 1000 µg/L. Especially, the lower metformin concentrations were measured in river waters containing percentages of conditioned wastewater from municipal wastewater treatment plants.

Results: Two complementary molecular biological methods for population analysis targeting the 16S rRNA gene regions *V*1–*V*3, i.e.: (1) 16S amplicon sequencing and (2) polymerase chain reaction (PCR) combined with denaturing gradient gel electrophoresis (DGGE). Both analyses demonstrated significant microbiome alterations even at low metformin concentrations being analysed in German rivers. The amplicon sequencing revealed the most distinct shifts in the *Firmicutes* phylum, or more specifically, within the *Bacillales* order, which were most affected by metformin exposure. Within the *Bacillales* order, the *Planococcaceae* family, which is described to provide essential amino acids for the fish, completely disappeared after metformin treatment. Conversely, the percentage of other bacteria, such as *Staphylococcaceae*, increased after exposure to metformin. Similarity profiles of the microbiomes could be generated using the Sørensen index calculation after PCR-DGGE analyses and confirmed shifts in the composition of the brown trout intestinal microbiome after metformin exposures. In vitro gene expression analyses of virulence factors from fish pathogens, previously identified in the fish microbiomes DNA extracts, were conducted in the presence or absence of environmentally relevant concentrations. Here, marker genes of *Enterococcus faecium*, *Enterococcus faecalis*, and *Aeromonas hydrophila* were detected and quantified via PCR approaches, firstly. An increased expression of the species-specific virulence genes was observed after normalisation with control data and ribosomal housekeeping genes.

Conclusion: Environmentally relevant concentrations of metformin can alter the composition in gut microbiome of brown trout in different ways. Both, the metformin-induced expression of virulence genes in fish pathogens in vitro and the impact of metformin on the microbiome composition in vivo in larval brown trout open the discussion about a possible long-term effect on the vitality, growth, and development in more mature brown trouts.

Keywords: Microbiome, Brown trout, Metformin, Virulence

Background

The ecological condition of most German river systems is in an insufficient shape, according to the most recent European Environment Agency assessment in 2018 [1]. In recent years, micro-pollutants such as industrial chemicals, pharmaceuticals, personal care products, and

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pesticides have become topics of interest and concern in the public and scientific community [2–4]. Even in low concentrations, these chemicals can affect surface water ecosystems and the health of aquatic organisms during constant exposure throughout their lifespan [5, 6]. The typical lifestyle in industrialised countries is rapidly enhancing the prevalence of diabetes type II [7–9]. This prevalence of diabetes is the reason for the consumption of metformin in industrialised countries, as it is the most widely prescribed anti-diabetic drug [10] and represents one of the most prescribed pharmaceuticals worldwide [11–14]. The defined daily dose (DDD) of metformin is 2 g. Metformin was prescribed with 590 Mio DDD [15] in Germany in 2015, resulting in a total prescribed amount of 1180 tons. Metformin cannot be metabolised by the human body and therefore is excreted unchanged [16]. However, metformin can change the composition of the intestinal microbiome of humans and rodents [17, 18].

In the past few years, the intestinal microbiome has received increasing attention amongst scientific researchers, as several studies revealed a strong correlation between the composition of the gut microbiome and the occurrence of certain diseases [19, 20]. The gut microbiome can interact with the host's brain via the microbiome–gut–brain–axis, and can even alter the behaviour of the host [21, 22]. Furthermore, the microbiome interacts with the host's immune system, with a balanced gut microbiome already known to be an important defence against pathogens [23]. The gut microbiome can be divided into two fractions, including an allochthonous microbiome in the faeces, and a biofilm-forming microbiome associated with the gut mucus. Changes in the mucus-associated microbiome can have a major impact on the health and immune system of the host organism. Consequently, an imbalance in the intestinal mucus-associated microbiome can adversely affect the health of host organisms [24].

It is known that metformin is not transformed or degraded during passage through the intestinal gastrointestinal tract of humans, with high concentrations of the drug reaching wastewater treatment plants (WWTPs). Although the elimination rates of metformin in WWTPs are > 90% [25, 26] via degradation to various transformation products [9, 11], significant quantities of metformin are still released to the aquatic environment and found in surface waters of neighbouring river systems at concentrations of about 1 µg/L [25–28]. But metformin was also measured in concentrations up to 9.8 µg/L in some German river systems [29]. In addition, the Swiss Ecotox Centre derived an annual average environmental quality standard (AA-EQS) of 156 µg/L for metformin in freshwater systems [30]. However, the effects of metformin on aquatic organisms, and therefore on contaminated ecosystems, are largely unknown. Therefore, we investigated the possible impacts of metformin on the gut microbiome of larval fish. The epithelium-associated microbial bacteria of gastrointestinal tracts are expected to play a major role in the interaction between eukaryotic and prokaryotic systems, which has already been previously described for fish, also for brown trout [31, 32]. This hypothesis of a possible impact of metformin on the microbiome on brown trout larvae was studied in exposure experiments with defined environmental concentrations of metformin.

Material and methods

Test organisms

The brown trout is a teleost fish and belongs to the *Salmonidae* family. It is an endemic fish in Europe and prefers to live in the upper region of streams in oxygen-rich and cool waters [33, 34]. The growth rate of brown trout is temperature-dependent [39]. The test organisms came from a commercial fish breeder (Forellenzucht Lohmühle, Alpirsbach-Ehlenbogen, Germany).

The experiments were conducted in strict accordance with German legislation and were approved by the animal welfare committee of the Regional Council of Tübingen, Germany (authorisations ZO 1/15 and ZO 2/16).

Metformin exposure of brown trout larvae

Metformin hydrochloride (99% purity; Sigma Aldrich, Munich, Germany) was used (CAS number: 1115–70–4). The substance was soluble in water, so a solvent was not necessary. The concentrations of metformin given in this paper refer to metformin as a pure substance and not as metformin hydrochloride.

The experiment was conducted as described in Jacob et al. [35]. At the beginning of the experiment, the trout larvae were in an eyed-egg stage, 48 days post-fertilisation (dpf). The larvae were exposed to five different treatments of metformin (0, 1, 10, 100, and 1000 µg/L real water concentrations) in triplicates at 7 °C in a climate chamber from 48 dpf until 8 weeks after yolk-sac consumption. In total, 210 fish larvae were studied for microbiome composition. The exposure of the eggs was conducted in a semi-static system in glass aquaria containing 10 L medium. Twice a week, 50% of the medium was exchanged with freshly prepared medium and excess food and faeces were removed. For the preparation of the medium, aerated, filtered tab water (iron filter, active char filter, particle filter) was used. Concerning the stability of the test, temperature, pH, oxygen content, and conductivity were monitored at the beginning and the end of the experiments (mean temperature = 7.16 °C ± 0.30 °C; mean pH = 8.05 ± 0.33; mean oxygen = 10.54 mg/L ± 0.16 mg/L; mean

conductivity = 410.43 µS/cm ± 20.29 µS/cm). Larval fish, 8 weeks after yolk-sac consumption, were euthanised with an overdose of tricaine methanesulfonate (MS 222; 1 g/L buffered by $NaHCO_3$) and a severance of spine. Gut samples were dissected with sterile equipment. Due to the small size of the fish, the gut was not divided by sections, but removed in total and stored at − 80 °C in sterile tubes.

DNA extraction for molecular biological analysis

The total DNA of samples from the fish guts, containing the microbiome and parts of the mucus such as epithelial DNA, was extracted from larval 156 days old trout, which were exposed to metformin for 108 days. With the exception of the faeces, a more distinct separation of eukaryotic from prokaryotic tissue/mucus types was not possible due to the larval age of the fish. For further investigation of the composition of the microbiome, the prokaryotic 16S ribosomal DNA (rDNA) was studied, and the eukaryotic DNA was discriminated via PCR primer specificity and/or databank alignments.

Faeces were removed from the dissected intestinal tract by gentle squeezing and washing with sterile water, to ensure only the mucus adherent microbiota were captured for characterisation [31]. Due to the young age of the fish, the guts were too small to rinse with peptone water for extracting the adherent bacteria, therefore the whole gastrointestinal tract was used [36]. The total DNA was extracted out of the autochthonous bacteria using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). The DNA was eluted in 50 µL of DES solution (pure water) and quantified via a NanoDrop ND-1000 spectrophotometer (Additional file 1: Table S1). The DNA of 2–3 extractions, depending on the gut sizes, was pooled due to the small amount of DNA obtained from single gut.

Denaturing gradient gel electrophoresis (DGGE)

The influence of metformin on the intestinal microbiome of the brown trout larvae was investigated by carrying out PCR-DGGE analyses, targeting the *V*1–*V*3 region of the eubacteria 16S rRNA genes with an amplicon size of 509 bp (base pair). In each PCR reaction, a 2.5 µL buffer (10×), 0.5 µL of deoxyribonucleotide triphosphate (dNTP; 10 µm), 0.25 µL of each primer (40 µm) (Additional file 1: Table S2), 0.125 µL of Taq polymerase, and 1 ng/µL template (10 ng DNA per sample) were used, and the volume was adjusted to 25 µL by adding water. The temperature profile consists of 3 min at 95 °C followed by 30 times at 95 °C for 30 s, 56 °C for 1 min, and 72 °C for 2 min. The prokaryotic 16S rDNA PCR amplicons were controlled by a 1% agarose gel electrophoresis, together with reference bacterial 16S rRNA gene amplicons, using SYBR Gold fluorochrome for DNA band visualisation.

The DGGE gel was made as described previously [37] with a linear urea gradient from 40 to 70%. Reference markers [*Escherichia coli* (DSM 1103), *Pseudomonas aeruginosa* (DSM 1117), *Enterococcus faecalis* (DSM 20478), *Enterococcus faecium* (DSM 20477), *Staphylococcus aureus* (DSM 2569), and *Stenotrophomonas maltophilia* (SMK279a)] were generated and used as a ladder to enable meaningful comparisons across different DGGE gels. A maximum of 15 µL of the PCR samples, containing approximately equal amounts of PCR product, were loaded onto the gel. The running time of the gel was 17 h at 70 volts and 56 °C. The DGGE gel was analysed via an F1 Lumi-Imager workstation (Roche Diagnostics, Mannheim, Germany) using the Lumi-Imager software (LumiAnalyst 3.1).

The SØrenson index (C_s) was used to compare metformin impacted microbiome composition with the control samples according to the DGGE band profiles, with values ranging between 0 (least similar) and 1 (most similar). With these values, it was possible to describe the similarity between two samples, ranging from 0 to 100% similarity. The following equation describes the C_s calculation:

$$C_s = \frac{2j}{(a+b)}, \tag{1}$$

where "*a*" represents the number of DNA bands in one lane, "*b*" the number of DNA bands in another lane, and "*j*" the number of common DNA bands within both lanes. Higher C_s values equate to a higher percentage of similarity.

16S rRNA gene amplicon sequencing

The DNA samples were given to Eurofins Genomics (Ebersberg, Germany) for investigation of the intestinal microbiome via 16S rRNA gene amplicon sequencing using MiSeq, with 2 × 300 bp targeting the *V*1–*V*3 16S rRNA gene region. In total, 90 DNA samples from gut microbiomes resulting from the sample pooling of the 210 fish guts were analysed. Here, a number of 18 DNA samples corresponded with a defined metformin concentration. The mean DNA concentration of the samples was 197 ng/µL (Additional file 1: Table S1), but 50 ng/µL was found to be optimal for amplicon sequencing. As a first quality control step of the microbiome analysis, all reads with ambiguous bases ("N") were removed. Chimeric reads were identified and removed based on the de novo algorithm of UCHIME [38], as implemented in the VSEARCH package [39]. The remaining set of high-quality reads was processed using minimum entropy decomposition (MED) [40]. MED provides a computationally efficient means to partition marker gene datasets into OTUs. Each OTU represents a distinct cluster with

significant sequence divergence relative to any other cluster. By employing Shannon entropy, MED uses only the information-rich nucleotide positions across reads, and iteratively partitions large datasets while omitting stochastic variation. The MED procedure outperforms classical identity-based clustering algorithms. Sequences can be partitioned based on relevant single nucleotide differences without being susceptible to random sequencing errors. This allows a decomposition of sequence datasets with a single nucleotide resolution. Furthermore, the MED procedure identifies and filters random "noise" in the dataset, e.g. sequences with a very low abundance (less than 0.02% of the average sample size). All reads were filtered with the default Illumina filter procedure (chastity filter). Paired-end reads were merged using the software FLASH (2.2.00 [41]). Pairs were merged with a minimum overlap size of 10 bp to reduce false-positive merges. To assign taxonomic information to each OTU, dc-megablast alignments of cluster representative sequences to the sequence database [Reference database: NCBI_nt (Release 2019-04-06)] were performed. The most specific taxonomic assignment for each OTU was then transferred from the set of best-matching reference sequences (lowest common taxonomic unit of all best hits). A sequence identity of 70% across at least 80% of the representative sequence was a minimum requirement for considering reference sequences. Investigations of the species or genus level was not intended, but could be performed in later analysis, if specific species-related enquiries were raised. Thus, a similarity cut off of 70% was sufficient for this analysis. Furthermore, the higher taxonomic levels of the bacterial population were investigated to have a kind of comparability between the 16S amplicon sequencing and the PCR-DGGE. The PCR-DGGE had a very limited resolution in the representation of the taxonomic levels. Further processing of OTUs and taxonomic assignments was performed using the QIIME software package (version 1.9.1, http://qiime.org/). Abundances of bacterial taxonomic units were normalised using lineage-specific copy numbers of the relevant marker genes to improve estimates [42]. The raw sequences are available at BioProject PRJNA524806.

In vivo detection of pathogens and virulence genes

The presence of facultative pathogenic bacteria and their virulence genes was investigated via standard PCRs in the brown trout microbiome DNA using bacteria-specific primers (Additional file 1: Table S3). Three exemplary pathogens were chosen, *Aeromonas hydrophila* (*A. hydrophila*) (DSM No. 30187), *Enterococcus faecalis* (*E. faecalis*) (DSM No. 20478), and *Enterococcus faecium* (*E. faecium*) (DSM No. 20477). The extracted DNA of the gastrointestinal tract of the brown trout was screened for the following virulence factors in *A. hydrophila*: aerolysin-related cytotoxic enterotoxin (act), heat-labile lipase (alt), heat-stable lipase (ast), and aerolysin (aerA). For *E. faecalis* and *E. faecium*, brown trout microbiomes were screened for the following virulence factors: *E. faecalis* antigen A (efaA), enterococcal surface protein (esp), collagen-binding adhesin (ace), aggregation substance (as), and gelatinase E (gelE).

Furthermore, quantitative polymerase chain reaction (qPCR) was also used to detect these facultative pathogenic bacteria, with the specific 23S rDNA primers for *E. faecalis/E. faecium*, and specific 16S rDNA primers for aeromonads (Additional file 1: Table S3). For this reason, calibration curves derived from the mentioned reference bacteria were used for quantification of so-called cell equivalents in mixed populations (Additional file 1: Figures S1, S2). The numbers of targets in each sample were derived using the corresponding calibration curve and normalised to 16S rDNA (cell equivalents per 16S rDNA copy number) [43]. Reactions were run in volumes of 20 µL, containing 10 µL of Maxima SYBR Green Master Mix (2×) (Thermo Scientific) and 8.2 µL of nuclease-free water (Ambion, Life Technologies, Karlsbad, Germany) of the respective primers (final concentration of 0.25 µm, Additional file 1: Table S3), and 1 µL template DNA (30 ng DNA per sample). Each sample was analysed in triplicate. The qPCR protocol comprised 3 min at 95 °C for activation of the DNA polymerase, followed by 40 cycles of 15 s at 9 °C and 30 s at 60 °C for primer annealing and elongation. To determine the specificity of amplification, a melting curve was recorded by raising the temperature from 60 to 95 °C (1 °C every 10 s). Data analysis was performed using the Bio-Rad CFX Manager software.

In vitro expression of virulence genes in facultative pathogenic bacteria

The influence of metformin on the gene expression of the aforementioned fish pathogens was investigated. These three facultative pathogens were cultured overnight with different metformin concentrations and 0 µg/L metformin as a control. The cultivation was carried out using Lysogeny broth (LB) (Carl Roth GmbH, Karlsruhe, Germany).

After cultivation up to an optical density (OD_{600}) of 1, the total RNA was isolated using the FastRNA® Pro Soil-Direct Kit (MP Biomedicals, Illkirch, France), according to the manufacturer's protocol (RNA concentrations are listed in Additional file 1: Table S4). Prior to reverse transcriptase (RT) reaction, digestion of DNA was accomplished using the TURBO DNA-free™ Kit (Ambion, Life Technologies, Karlsbad, Germany), according to the manufacturer's protocol. For subsequent RT reaction,

the TaqMan Gold RT-PCR Kit (Applied Biosystems, Life Technologies, Karlsbad, Germany) was applied. Solutions were prepared on ice by the addition of 5 µL of 10× TaqMan RT buffer, 11 µL of $MgCl_2$ (25 mm), 10 µL of dNTP mixtures (2.5 mm of each nucleotide species), 2.5 µL of random hexamers (50 µm), 1 µL of RNase inhibitor (20 u/µL), and 1.25 µL of MultiScribe reverse transcriptase (50 u/µL). A concentration of 30 ng/µL RNA was used for each sample. The reverse transcription was performed in a thermocycler with the following temperature profile: 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min.

Subsequent qPCR reactions for virulence gene quantification were run in volumes of 20 µL, containing 10 µL Maxima SYBR Green Master Mix (2×) (Thermo Scientific, Frankfurt, Germany) and 8.2 µL of nuclease-free water (Ambion, Life Technologies, Karlsbad, Germany) of the respective primers (final concentration of 0.25 µm, Additional file 1: Table S3), and 1 µL of the gained complimentary DNA. Each sample was measured three times. The qPCR protocol comprised 3 min at 95 °C for activation of the DNA polymerase, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C for primer annealing and elongation. To determine the specificity of amplification, a melting curve was recorded by raising the temperature from 60 to 95 °C (1 °C every 10 s) (Additional file 1: Figure S3). Data analysis was performed by using the Bio-Rad CFX Manager software and the normalised expression mode with ΔΔCt. For normalisation of the measured expression levels, reference genes were used [44, 45]. For the reference gene for *E. faecalis* and *E. faecium*, a specific region of the 23S rRNA gene was chosen, and for *A. hydrophila*, a specific 16S rRNA primer pair was used as a reference (Additional file 1: Table S3).

Statistical analysis

For the statistical analysis, all results were tested for a normal distribution using the Shapiro–Wilk test and the *p*-values were calculated using OriginPro (OriginLab Corp., Northampton, USA). The Student's *t* test was used if the data were normally distributed, to identify significant differences between the treatments. In the case of a non-standard distribution, the significance was tested by a Mann–Whitney *U*-test [46]. The significance of the changes in the Sørensen index in comparison to the control was investigated with the two-sample *t* tests on the α-levels of 0.05 and 0.01. The same statistical analysis was performed for testing the significance of the changes in the expression of the virulence genes. To test the significance of the shifts in the intestinal microbiome of the brown trout larvae nested analysis of variance (ANOVA) tests were performed using the metformin concentrations as nesting factor on the α-levels of 0.05 and 0.01. The statistical tests used and the *p*-values are presented in the following chapters. In consultation for the statistical analysis of the 16S amplicon sequencing α-diversity indices (Chao1, Shannon and Simpson) and β-diversity analysis (PCoA) were generated by Eurofins Genomics (Ebersberg, Germany) using the QIIME software package (version 1.9.1, http://qiime.org/) [47, 48]. For these analyses the results were normalised on the minimum sequence count of 32,325. The results of the weighted UniFrac PCoA can be found in Additional file 1: Figure S4. It was described before that the effects of metformin treatment on gut microbial composition were poorly captured by multivariate analysis [17].

Results and discussion

Impact of metformin on the microbiome

In order to investigate the mucus-associated microbiome, the population analyses rely on two independent molecular biological methods targeting fractions of the prokaryotic 16S rRNA genes [49]. Amplicon sequencing was applied to achieve this, targeting the *V*1–*V*3 variable regions of the 16S rRNA gene. During amplicon sequencing up to a maximum read length of 490 bp, gained sequences were amplified and sequenced for further bioinformatic evaluation. The PCR-DGGE, which was also targeting the 16S rRNA gene, was used for population analysis as an alternative method. The amplicon sequencing and the PCR DGGE were based on different principles to study whole bacterial populations targeting the *V*1–*V*3 16S rDNA region, as recommended by Wang et al. [49] to run different methods for population analysis of intestinal microbiomes.

Using the 16S amplicon sequencing for each metformin concentration and controls, 18 DNA samples for each metformin concentration from 210 individual fish were analysed. A total of 9,046,627 reads remained in the evaluation after quality filtering. All of these reads have passed the default Illumina filter procedure (chastity filter). The mean quality score was 32.10. From these 5,190,914 reads were assigned to 2549 operational taxonomic units (OTUs), and 99.4% of these OTUs were assigned to bacterial taxa.

The statistical diversity of the bacterial community in the samples was analysed with different α-diversity indices, i.e. Chao1, Shannon, and Simpson (Table 1). The number of observed species increased at 1 µg/L metformin to 120.15 ± 14.47 in comparison to the control, but declined at 10 µg/L metformin to 95.67 ± 11.83. The species numbers increased again at 100 µg/L and 1000 µg/L metformin to 112.43 ± 23.21. The Chao1- and Shannon-index showed the same pattern as the number of observed species. The Simpson's index showed a slight deviation in comparison to the control of 0.96 ± 0.005 at

Table 1 Average observed species, average Chao1, average Shannon, and average Simpson diversity indices (± standard error) for the different metformin treatments in the microbiomes of the 156 days old brown trout larvae

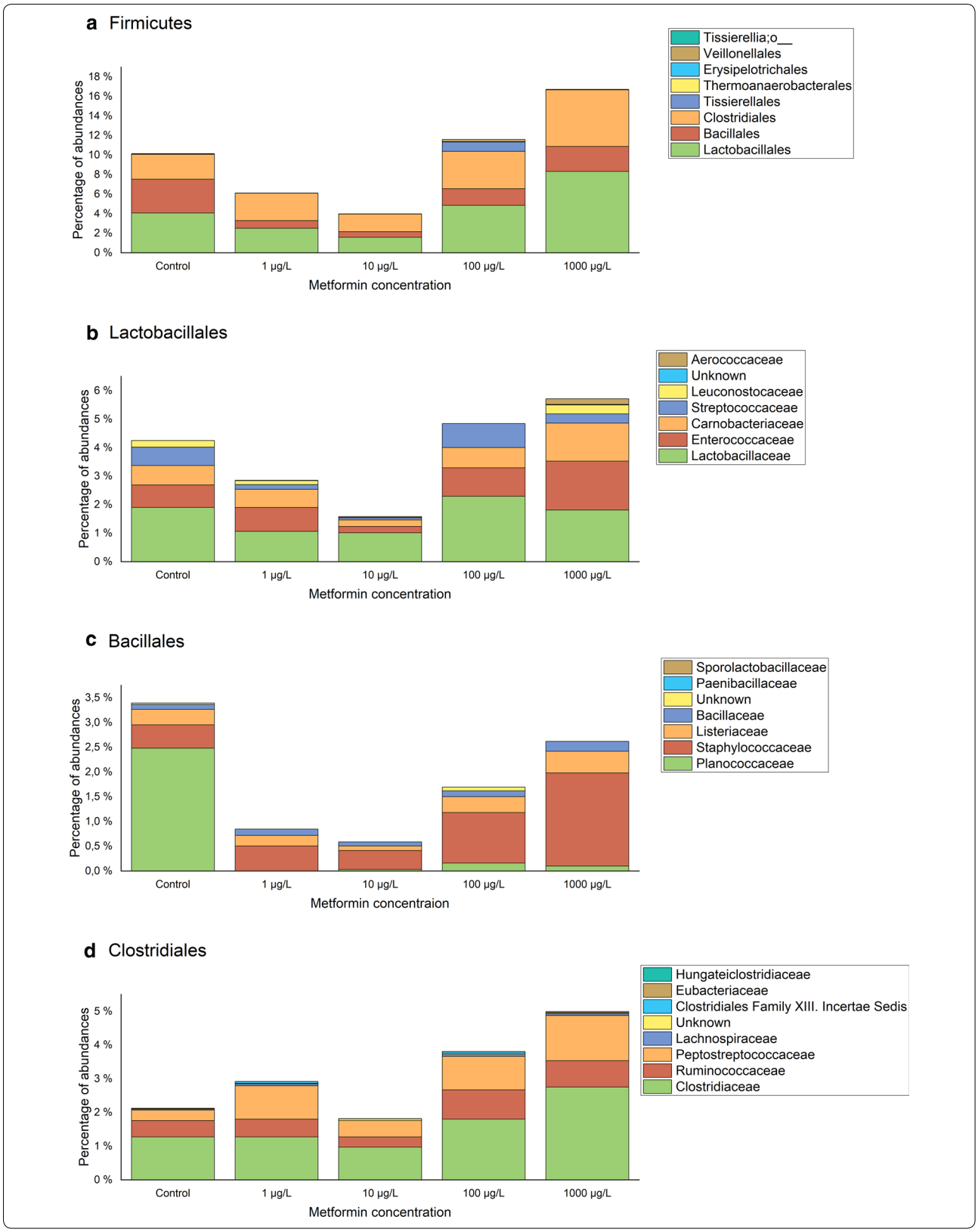
Metformin concentration	Observed species	Chao1	Shannon	Simpson
Control	101.18 ± 12.08	103.14 ± 12.30	5.57 ± 0.18	0.96 ± 0.005
1 µg/L	120.15 ± 14.47	122.12 ± 14.96	5.58 ± 0.20	0.95 ± 0.009
10 µg/L	95.67 ± 11.83	97.46 ± 12.24	5.43 ± 0.24	0.96 ± 0.007
100 µg/L	97.19 ± 12.50	99.11 ± 13.10	5.56 ± 0.16	0.96 ± 0.006
1000 µg/L	112.43 ± 23.21	114.25 ± 23.65	5.63 ± 0.32	0.96 ± 0.011

1 µg/L metformin with 0.95 ± 0.007. But no significant changes in the diversity between the metformin treatments were observed. Each statistical evaluation underlines the overall comparability of the analyses with the different metformin concentrations. The α-diversity indices showed that the overall count of the species and the diversity was unchanged by the metformin treatments. But the indices do not describe the abundance of different bacterial taxons. Therefore, the comparability among the different experimental designs is given and it was demonstrated that the shifts in the microbiome composition were caused by the presence of different metformin concentrations especially at lower taxonomic ranges.

The dominant phyla in the intestinal microbiome of the brown trout identified in the control samples were *Proteobacteria* (44%), *Actinobacteria* (41%), and *Firmicutes* (10%). These results are in agreement with other studies focussed on the gut microbiomes of freshwater fish [50]. More distinct changes in the intestinal microbiome composition of the fish, especially in the *Firmicutes* (ANOVA, $p \leq 0.01$), were observed at the order taxonomic level (Fig. 1a). The percentage of the entire phylum *Firmicutes* decreased from nearly 10% to 6.6% at the lower metformin concentrations of 1 µg/L and 10 µg/L, respectively. The percentage increased back to 15% at the higher metformin concentrations of 100 µg/L and 1000 µg/L. Small changes within other phyla could also be seen, but the changes within *Firmicutes* were more distinct. Therefore, we targeted the phylum *Firmicutes* as the focus of amplicon sequencing during this study. The dominant bacterial orders found in the *Firmicutes* phylum include the *Lactobacillales* (4%), *Bacillales* (3.5%), and *Clostridiales* (2.5%). Proportions of the *Lactobacillales* (ANOVA, $p \leq 0.05$) showed specific shifts at different metformin concentrations, decreasing at low metformin concentrations to 2.5% at 1 µg/L and 1.5% at 10 µg/L but recovering at higher metformin concentrations of 100 µg/L to 4.8% and 1000 µg/L to 8.3% (Fig. 1b, c). The changes in *Bacillales* showed the same trend but the changes in this order were not significant. Percentages of the *Lactobacillales* at high metformin concentrations of 100 and 1000 µg/L were found to be greater than percentages measured in the control samples (Fig. 1b). In contrast, percentages of the *Clostridiales* (ANOVA, $p \leq 0.01$) progressively increased after metformin exposure from 2.5% in the control to 2.8% at 1 µg/L, 3.8% at 100 µg/L, and to 5.8% at 1000 µg/L metformin (Fig. 1d). The 16S rDNA amplicon sequencing revealed a shift in the gut microbiome populations at different taxonomic ranks. These results expand upon previous analyses and published data, which focussed specifically on the *Firmicutes* phylum [35]. In extension we analysed shifts in lower taxonomic ranks, which more clearly illustrate the specific impact of metformin on the intestinal microbiome of the fish (Fig. 1). The effects of an increasing metformin concentration on the *Bacillales* order are illustrated in Fig. 1c. Since the *Planococcaceae* (ANOVA, $p \leq 0.05$) family disappeared from 2.5% in the control to under 0.1% after metformin treatment, the impact of metformin on this order is clear. The disappearance of the *Planococcaceae* is described to occur during ageing of the African freshwater killifish and was directly correlated with their ability to hydrolyse gelatine to produce essential amino acids [51]. Hence, the disappearance of *Planococcaceae* in trout microbiomes is hypothesised to impact the fish metabolism and may therefore influence the long-term vitality or fish development. In fact, the brown trout is supposed to get assess to most of their relevant nutrients via the digestions of their prey, especially essential amino acids. Nevertheless, it has to be assumed that the bioavailability of such nutrients is

(See figure on next page.)

Fig. 1 Composition of the phylum *Firmicutes* (**a**). Different orders of the phylum *Firmicutes*, i.e. *Lactobacillales* (**b**), *Bacillales* (**c**), and *Clostridiales* (**d**). Percentages are in comparison to the overall intestinal mucoid microbiome composition. Changes in the intestinal microbiome within these taxonomic units, after exposure to metformin concentrations ranging from 0 µg/L (control) to 1000 µg/L, were analysed with 16S rRNA gene amplicon sequencing after 108 days of exposure


a Firmicutes
Percentage of abundances
18 %
16 %
14 %
12 %
10 %
8 %
6 %
4 %
2 %
0 %
Control
1 µg/L
10 µg/L
100 µg/L
1000 µg/L
Metformin concentration
Tissierellia;o__
Veillonellales
Erysipelotrichales
Thermoanaerobacterales
Tissierellales
Clostridiales
Bacillales
Lactobacillales
b Lactobacillales
Percentage of abundances
6 %
5 %
4 %
3 %
2 %
1 %
0 %
Control
1 µg/L
10 µg/L
100 µg/L
1000 µg/L
Metformin concentration
Aerococcaceae
Unknown
Leuconostocaceae
Streptococcaceae
Carnobacteriaceae
Enterococcaceae
Lactobacillaceae
c Bacillales
Percentage of abundances
3,5 %
3,0 %
2,5 %
2,0 %
1,5 %
1,0 %
0,5 %
0,0 %
Control
1 µg/L
10 µg/L
100 µg/L
1000 µg/L
Metformin concentraion
Sporolactobacillaceae
Paenibacillaceae
Unknown
Bacillaceae
Listeriaceae
Staphylococcaceae
Planococcaceae
d Clostridiales
Percentage of abundances
5 %
4 %
3 %
2 %
1 %
0 %
Control
1 µg/L
10 µg/L
100 µg/L
1000 µg/L
Metformin concentraion
Hungateiclostridiaceae
Eubacteriaceae
Clostridiales Family XIII. Incertae Sedis
Unknown
Lachnospiraceae
Peptostreptococcaceae
Ruminococcaceae
Clostridiaceae

impacted by the presence of specific bacteria involved in digestion processes in the gut microbiome. The bacteria in the intestinal microbiome of the carnivorous fish are a contributor to the enzyme activity in the gut. Depending on the host's trophic level, the activity of digestive enzymes can be different [52]. For example, it has been shown that the genome of herbivorous mammals does not contain endogenous genes coding cellulose-digesting enzymes, and they are only able to digest cellulose due to *Clostridia* species in their microbiome. Recent studies have shown the same host–guest relationship in different fish [50, 53]. The disappearance of specific bacteria, which are essential for the digestion of food, can have a major impact on the health and development of the host. In contrast to the *Planococcaceae*, the abundance of *Staphylococcaceae*, which contain facultative pathogens causing enteritis [19], increased in a metformin concentration-dependent manner from 0.5% in the control to 1.9% at 1000 µg/L metformin. There was also a distinct impact on the *Lactobacillales*, e.g. for the *Enterococcaceae* (ANOVA, $p \leq 0.05$), which also contain facultative pathogenic bacteria. For the *Lactobacillales*, the population analysis showed firstly a decrease in abundance at low metformin concentrations, followed by an increase at higher metformin concentrations (Fig. 1b).

We also found bacterial families whose abundances were not affected by metformin concentrations (e.g. the *Bradyrhizobiaceae* and *Hyphomicrobiaceae*). These two families belong to the *Rhizobiales*, which constitutes 20–40% of the overall intestinal microbiome and therefore, represents one of the most abundant orders found in the mucoid intestinal microbiome in this study.

As previously published, a change in carbohydrate metabolism was described in these larval brown trout due to exposure to metformin, but no lethal effect or strong change in health was observed in 156 days old fish larvae [35]. However, the results of the 16S rRNA gene amplicon sequencing revealed that metformin specifically altered the intestinal microbiome of the larval brown trout in a distinct way. One of the most abundant bacterial orders, the *Rhizobiales*, exhibited negligible changes in abundance during exposure to high metformin concentrations. On the other hand, other taxonomic groups in the fish microbiome, such as the *Planococcaceae*, disappeared. The impact of metformin on important bacterial orders or families for the processing of nutrition, such as the *Lactobacillales*, is clearly visible (ANOVA, $p \leq 0.05$). Furthermore, distinct changes of the microbiome due to metformin treatment were also observed in the great ramshorn snail (*Planorbarius corneus*) in an additional experiment (data not shown). In this aquatic pulmonate gastropod mollusc the abundance of the *Lactobacillales* showed also a decrease at low and an increase at higher metformin concentrations. Whereas the *Proteobacteria* showed no distinct changes.

Community similarity indices impacted by metformin

A number of 18 DNA samples from fish mucus gastrointestinal tracts were investigated by PCR-DGGE for each metformin concentration. The DGGE band pattern of amplified 16S rRNA genes revealed differences in the generated profiles, which were specific for each metformin concentration in comparison to the control. The total number of DNA bands depends on their AT/GC content of the amplified *V*1–*V*3 region. Figure 2 shows an example of a DGGE analysis with different amplicons and therefore diverse DNA band profiles.

The total number of DNA bands in microbiome samples exposed to different metformin treatments exhibited no significant change in comparison to the control. The average number of bands was 13.63 ± 2.78 in the control, and the highest deviation from the control (12.50 ± 1.46 DNA bands) was recorded in the 1000 µg/L metformin treatment. However, the average number of identical bands (i.e. value "*j*" of Eq. 1) between the control and metformin-treated microbiome samples decreased in a concentration-dependent manner, from 10.67 ± 1.49 in the control samples to 3.50 ± 1.01 at 1000 µg/L metformin concentration (Additional file 1: Table S5).

Calculation of the Sørensen index (Eq. 1, see "Material and methods"), which facilitates a comparison of the similarity of two individual samples, confirms these observations. There is a significant change in the similarity of the mucoid intestinal microbiome composition with typical

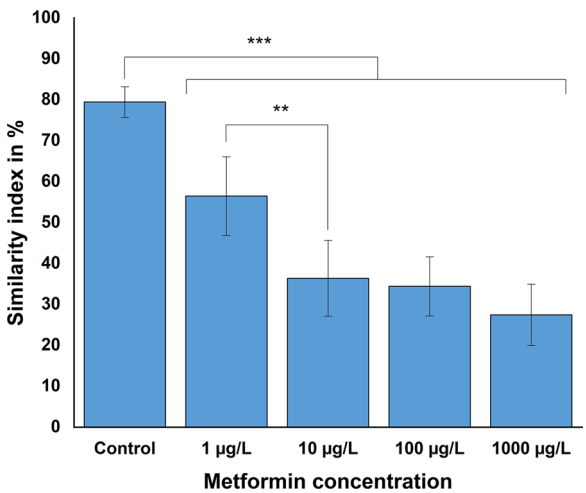


Fig. 2 An exemplary image of a DGGE gel of the mucoid intestinal microbiome of brown trout larvae treated with different metformin concentrations [ladder, control (a), 1 µg/L (b), 10 µg/L (c), 100 µg/L (d), and 1000 µg/L (e)]. The ladder represents a mixture of different bacteria-specific amplicons

environmentally occurring metformin concentrations of 1 µg/L and 10 µg/L, compared to the controls (Fig. 3). While the similarity of the controls ($n=18$) among the intestinal microbiome was around 80% (without any metformin), the Sørensen index dropped to 56% at a metformin concentration of 1 µg/L (t-test, $p \leq 0.01$) and to 35% at a metformin concentration of 10 µg/L (t-test, $p \leq 0.05$) in comparison with the control samples, which was calculated as statistically significant. The treatment with 100 µg/L and 1000 µg/L metformin resulted in a similarity index of about 30%, which seems to describe a more stable composition at higher metformin concentrations (Fig. 4). Nonetheless, it is significantly different to the control samples.

The results of the PCR-DGGE analysis confirm an impact of metformin on the bacterial composition of the mucus-associated microbiome of the brown trout. Different bacterial species may disappear, whilst other species may benefit from elevated metformin concentrations and exhibit increased growth rates. These dynamic variations alter the band profiling and result in a different Sørensen index calculation. Therefore, it is important to have two analyses to comprehensively verify possible population shifts in microbiomes, as recommended by Wang et al. [49]. When combined, they give a comprehensive insight into variations in the different ranks of taxonomic and population composition.

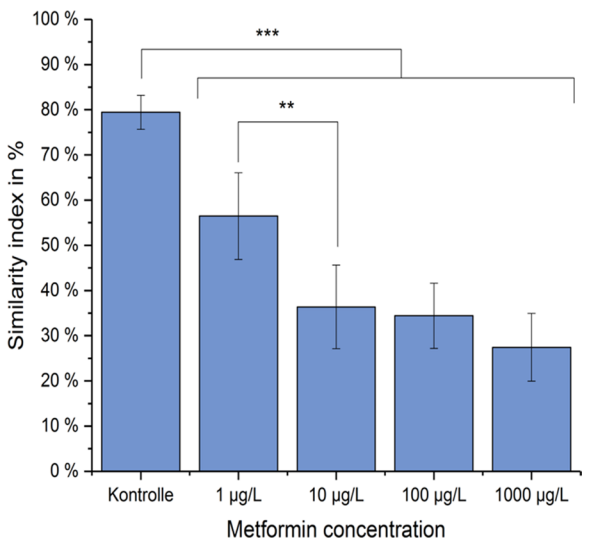


Fig. 3 Sørensen index calculated from DGGE profiles from metformin exposed microbiome extracts. Significant changes are illustrated by using the Student's t-test evaluation ($n=18$ samples for each concentration); ***$p \leq 0.01$; **$p \leq 0.05$

In vivo detection of fish pathogens and in vitro expression of their virulence genes

The presence of the facultative pathogenic bacteria *A. hydrophila*, *E. faecalis*, and *E. faecium* as part of gastrointestinal tract was investigated in vivo by using a standard PCR, followed by agarose gel electrophoresis. The facultative pathogenic bacteria were detected in nearly all microbiome DNA samples, including trout from rivers, fish farms, and larval trout from aquarium experiments. Besides the taxonomic gene markers, most virulence genes were identified in the microbiome samples (controls and metformin-treated microbiomes) of caged fish and the free-living brown trout. For *A. hydrophila*, brown trout microbiomes were screened for the virulence genes aerolysin-related cytotoxic enterotoxin (*act*), heat-labile lipase (*alt*), heat-stable lipase (*ast*), and aerolysin (*aerA*) and for *E. faecalis/E. faecium* the virulence genes *E. faecalis* antigen A (*efaA*), enterococcal surface protein (*esp*), collagen-binding adhesin (*ace*) as aggregation substance, and gelatinase E (*gelE*) were targeted. In contrast, not all investigated species-specific virulence factors were present in the DNA extracts from the fish gastrointestinal microbiomes, as indicated in Table 2.

In addition to standard PCR, the in vivo abundance of the pathogens was also analysed by qPCR. The amplification curve and melting point calculation of the amplicons are presented in Additional file 1. In this study, relative quantification based on 16S rDNA copy numbers was used [43]. Both, enterococci and aeromonads were detected in the microbiome of all 90 DNA extract samples including nine environmental microbiome fish samples (river), and microbiome samples from the fish farm ($n=3$). The qPCR results showed the highest abundance of *A. hydrophila* in the microbiomes of fish from the fish tanks, followed by the fish microbiomes from the river and the lowest abundance in the microbiome samples from the fish farm. The measured abundance of *E. faecalis* and *E. faecium* was the highest in the microbiome samples from the fish farm, followed by the fish tank and the lowest abundance in microbiome samples from the river. The fact that the fish pathogens were also detected in the microbiomes of the brown trout larvae

(See figure on next page.)

Fig. 4 Expression of virulence genes in *Enterococcus faecium* (**a**), *Enterococcus faecalis* (**b**), and *Aeromonas hydrophila* (**c**) after exposure with metformin. Bacteria-specific virulence genes and applied metformin concentration are given on the x-axis. The expression factors were normalised to housekeeping genes and the untreated control for standardisation. The significance was tested using the Student's t-test for *E. faecalis*, *E. faecium* (*$p \leq 0.05$; **$p \leq 0.01$) and the Mann–Whitney U test for *A. hydrophila* (*$p \leq 0.05$)

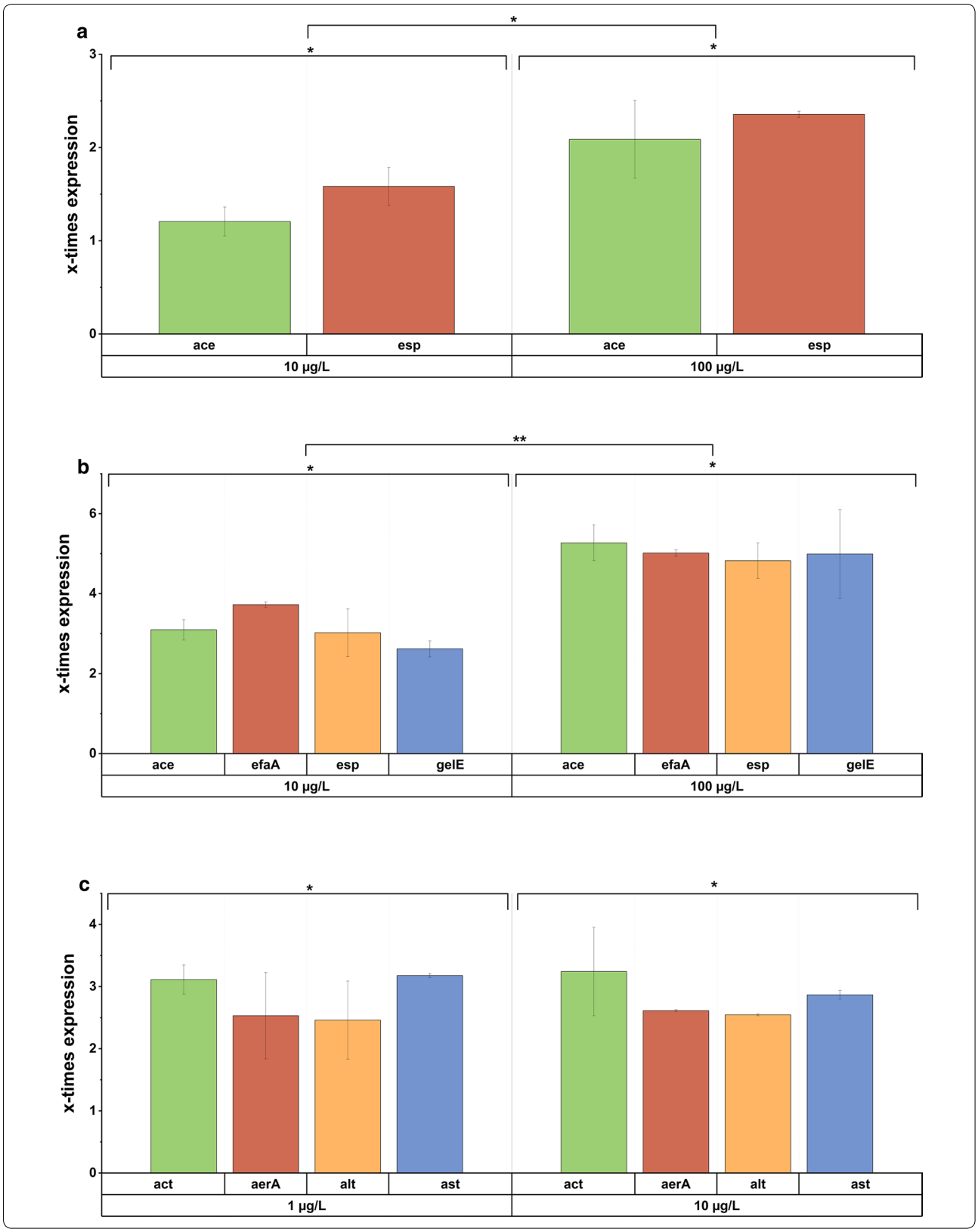

a
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Table 2 PCR-based results detecting fish pathogen species and their species-specific virulence genes in microbiome DNA samples from different habitats (fish tank, river, fish farm)

Standard PCR	Fish tank		River		Fish farm	
	Detected	Samples	Detected	Samples	Detected	Samples
E. faecium/E. faecalis	+	16/18	+	3/3	+	3/3
esp	+	15/18	+	3/3	+	3/3
gelE	+	12/18	+	3/3	+	3/3
efaA	+	5/18	+	3/3	+	3/3
ace	+	1/18	−	0/3	−	0/3
as	−	0/18	−	0/3	−	0/3
A. hydrophila	+	17/18	+	3/3	+	3/3
act	+	5/18	+	3/3	+	3/3
alt	+	16/18	+	3/3	+	3/3
ast	+	10/18	+	3/3	+	3/3
aerA	+	13/18	+	3/3	+	3/3
qPCR	**Fish tank ($n=90$) Copy numbers/16S rDNA**		**River ($n=3$) Copy numbers/16S rDNA**		**Fish farm ($n=3$) Copy numbers/16S rDNA**	
E. faecium/E. faecalis	8.16E−04 ± 6.41E−04		3.52E−04 ± 3.43E−04		6.20E−02 ± 4.79E−05	
A. hydrophila	1.69E−01 ± 5.09E−02		5.52E−02 ± 5.93E−02		7.35E−03 ± 4.83E−03	

The number of investigated fish samples is given together with the target-specific positive tested fish samples. The fish pathogens in microbiome DNA extracts were also quantified by qPCR including standard deviation (bottom part of the table)

from laboratory fish tanks may be attributed to the fact that the fish eggs originating from a fish breeder were not sterile before the exposure experiment start. Hence, it is supposed that the fish pathogens were attached to the egg surfaces. The larval stage of the fish might also contribute to the high abundance of the fish pathogens, because the development of their immunity is still ongoing and might not be able to adequately response to the pathogens in this stage of the fish [54]. The standard deviation equals in many cases the given value following the fact that these are a mean value of measurements from biological samples (Table 2).

The standard PCR method showed the presence of the facultative pathogenic bacteria *A. hydrophila*, *E. faecalis*, *E. faecium*, and their virulence genes in the investigated samples. The qPCR method revealed the abundance of aeromonads and *E. faecalis*/*E. faecium* in a quantitative analysis. However, the fish pathogens and their specific virulence factors were not only detected in samples from this study, but also in environmental samples from brown trout using both methods. As a consequence, gene expression analyses were performed in vitro on previously positively identified virulence factors from DNA-based detection for enterococci and aeromonads with the original microbiomes.

In vitro gene expression analyses of virulence factors in fish pathogens

The gene expression in the identified facultative pathogenic bacteria in the presence or absence of metformin was studied in vitro. Due to the low abundance of the targets for RNA-based analysis in the original microbiomes, gene expression experiments were performed in vitro with bacteria cultures. These cultures were exposed to metformin concentrations close to environmentally occurring concentrations [26, 28, 29, 55]. Here the impact of metformin on the expression of virulence genes of pathogens could be demonstrated (Fig. 4). The expression levels were investigated by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The analysed expressions were normalised on a 16S rRNA housekeeping gene and the untreated control sample of each target. And the growth rate of the control and the metformin supplemented cultures were investigated, but showed no significant differences among the metformin treatments. In the bacterial culture for *E. faecium*, only the *ace* and *esp* virulence genes were studied. In response to metformin treatment, the expression of these two virulence genes increased in a concentration-dependent manner up to two to three times relative to the expression of the control samples. The changes in the expression were all significant (t-test, $p \leq 0.05$) compared to the control (Fig. 4a). The four investigated virulence genes (*ace*, *efa*A, *esp*, and *gel*E), being specific to *E.*

faecalis, were expressed three times higher in the cultures supplemented with 10 µg/L metformin and 5 times higher in the cultures at metformin concentrations of 100 µg/L. There was also a concentration-dependent increased expression compared to the control (*t*-test, $p \leq 0.01$) for *E. faecalis* (Fig. 4b). In *E. faecalis* and *E. faecium*, virulence factors such as *efaA* serve as surface proteins to adhere on cell walls of the hosts, whilst *esp*, *ace*, and *gelE* serve as aggregation substances or surface proteins, which allow the bacteria to avoid the host's immune system. Four virulence genes specific for *A. hydrophila* were studied, including the genes, coding for toxins, *act*, *aer*A, and the two lipase coding genes *alt* and *ast*. All these virulence genes allow the pathogens to adhere to the epithelial cells of the gut and to associate with the intestinal mucus. They were expressed three times higher at 1 µg/L and 10 µg/L metformin (Mann–Whitney *U*-test, $p \leq 0.05$), compared to the controls (Fig. 4c). These virulence factors in *A. hydrophila* promote the invasion of pathogens into the host.

In fish pathogens, the virulence factors were higher expressed in bacteria within media supplemented with metformin compared to controls. These results indicate that metformin possesses a direct impact on the virulence potential of the tested fish pathogens. The virulence factors could pass or adhere to the gut epithelium and directly influence the immune status of the fish. This altered expression of virulence genes showed that pharmaceuticals even at lower environmental concentrations can have an influence on the expression levels of genes in the intestinal microbiome. Hence, such pollutants might serve as an exogenous stimulus which may influence different pathways by activation or inhibition in the bacteria. In this case, an increased virulence, as an example, could be an advantage for survivability and persistence for the pathogens. Other microbial responses might also be expected and could lead, e.g. to an altered communication via quorum sensing within the ecosystem "biofilm". It should be mentioned, however, that fish in the environment are exposed to a number of environmental and man-made stressors (e.g. chemical, temperature, nutrients, etc.). Interactions with several stressors might result in synergistic effects, which are expected to be stronger than from either of the stressors alone [56]. These stress factors also have an impact on the immune system of the fish, leading to an increased pathogenicity, and ultimately, in enhanced mortality rates [57, 58]. Actually, the fish in environmental habitats are exposed to a number of pharmaceuticals, which might also have an influence on the fish microbiomes. In a recent study in the German rivers Rhine and Saar up to 61 analytes were found, some of them with known biotoxicity, but mostly with unknown influence on the environment and particularly on the bacterial microbiomes [59]. The present results of our study combined with detected surface water metformin concentrations of up to 9.8 µg/L in some German river systems [29] lead to the conclusion that the health of free-living fish could also be adversely impacted by metformin and this might have a long-term relevance for the lack of recovery in fish population in aquatic systems.

Conclusion

Environmental concentrations of metformin can alter the composition in gut microbiome of brown trout larvae in different ways. Both the 16S amplicon sequencing and the PCR-DGGE analyses demonstrated a significant population shift in specific bacterial orders and families even at different environmentally relevant metformin concentrations. Therefore, the observed population changes together with the increased expression of virulence factors make sub-lethal impacts of metformin on brown trout larvae evident. It should be noted, however, that the fish showed no lethal effect or strong change in health during their larval growth phases and during the exposure to only metformin for a short time period. It is still unclear whether a long-term exposure of the maturing fish to metformin concentrations might lead to more severe health effects, such as altered immune systems, growth development, fertility, and metabolic syndromes. Nevertheless, it was shown that one pharmaceutical can have an impact, even in low concentrations, on the composition and activity of fish larvae microbiomes. The impacts of more complex mixtures of anthropogenic chemicals released into the aquatic environment on microbiomes in aquatic organisms should be studied for a more comprehensive risk characterisation. Microbiome studies should be discussed to be implemented in future monitoring and toxicity testing in regulatory chemical management as a follow up of the fish toxicity test. The DNA-based investigations of the intestinal microbiomes via next generation sequencing is designed for high throughput screenings and thus able to analyse samples in a large scale. Nevertheless, further investigations on the interaction between host and microbiome in aquatic organisms need to be done to make defined conclusions about the effects of chemicals on the intestinal microbiome and vitality of the host.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12302-020-00341-6.

Additional file 1. Additional Tables S1–S5 and Figure S1–S4.

Abbreviations
AA-EQS: Annual average environmental quality standard; *A. hydrophila*: *Aeromonas hydrophila*; ace: Collagen-binding adhesin; act: Aerolysin-related cytotoxic enterotoxin; aerA: Aerolysin; alt: Heat-labile lipase; as: Aggregation substance; ast: Heat-stable lipase; bp: Base pair; DDD: Defined daily dose; DGGE: Denaturing gradient gel electrophoresis; dNTP: Deoxyribonucleotide triphosphate; dpf: Days post-fertilisation; *E. faecalis*: *Enterococcus faecalis*; *E. faecium*: *Enterococcus faecium*; efaA: *E. faecalis* antigen A; esp: Enterococcal surface protein; gelE: Gelatinase E; LB: Lysogeny broth; MED: Minimum entropy decomposition; OD_{600}: Optical density; OTU: Operational taxonomic unit; PCR: Polymerase chain reaction; qPCR: Quantitative polymerase chain reaction; rDNA: Ribosomal DNA; RT: Reverse transcriptase; WWTP: Wastewater treatments plants.

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Authors' contributions
ER performed the microbiome analyses and the statistical analyses. TS supervised the microbiome analyses and designed the study. SJ performed the exposure experiment. RT supervised the exposure experiment. All authors read and approved the final manuscript.

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Data availability
Raw sequences from the 16S Amplicon sequencing are available at the NCBI database BioProject PRJNA524806.
The authors declare that the data supporting the findings of this study are available within the paper and its Additional file 1. Additional information is available at ISMEJ's website.

Ethics approval and consent to participate
The experiments were conducted in strict accordance with German legislation and were approved by the animal welfare committee of the Regional Council of Tübingen, Germany (authorisations ZO 1/15 and ZO 2/16).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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