Passive exposure to cannabidiol oil does not cause microbiome dysbiosis in larval zebrafish

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ABSTRACT

The use of cannabidiol oil derived products has dramatically increased in popularity and is predicted to grow steadily over the next decade. Given its relative stability, cannabidiol is likely to accumulate in the environment and affect aquatic animals and their host-associated microbiomes. Here, using zebrafish larvae, a model system in environmental toxicology, we show that passive exposure to a concentration as high as 200 μg/L cannabidiol oil did not affect larvae survival and had limited effects on their host-associated microbial communities. We found that the changes in community structure were limited to a decrease in two sequence variants identified as *Methylobacterium-Methylorubrum* sp. and one ASV identified as *Staphylococcus* sp., as well as the increase of one sequence variant identified as *Chryseobacterium* sp., a bacterium commensal to zebrafish. More importantly, we found that cannabidiol oil did not affect the overall richness and diversity of the exposed fish microbiomes. These results suggest that passive exposure to cannabidiol oil is unlikely to impact aquatic organisms in significant ways.

0 Importance

Cannabidiol, one of the most important active chemicals found in cannabis, is increasingly used in over-the-counter products. Like many other household products, including illicit drugs, cannabidiol ultimately finds its way into our waterways. Such pollution can have dramatic impacts on aquatic organisms, especially those that depend on aquatic ecosystems for their reproduction and early development. Environmental pollution often affects organisms by disrupting the organism's important microbiome, which regulates many physiological functions and acts a defense mechanism against infections. The significance of our research is to show that exposure to clinically relevant concentrations of cannabidiol oil does not disrupt the zebrafish larvae microbiome and did not negatively affect the survival of the animal at a crucial developmental stage.

1. Introduction

Cannabidiol (CBD) along with Δ9-tetrahydrocannabinol (THC) are the most important phytocannabinoids in *Cannabis sativa* plants (Andre et al., 2016; Bonaccorso et al., 2019). Cannabidiol interacts with the central nervous system via the endocannabinoid system (Rong et al., 2017; Thomas et al., 2007), which is linked to memory, fertility,

appetite, and stress response in humans, among other things (Rong et al., 2017; Russo, 2016). For this reason, cannabidiol is now considered for therapeutic use for attenuating symptoms caused by a variety of conditions, including cancer, epilepsy, central sensitivity syndromes, anxiety disorders, and autoimmune diseases such as multiple sclerosis (MacCallum and Russo, 2018; Maurya and Velmurugan, 2018).

As of 2020, the U.S. Food and Drug Administration only approved a single drug with cannabidiol as the main active ingredient, Epidiolex used for the treatment of epilepsy (MacCallum and Russo, 2018). Yet, the sale of cannabidiol oil as dietary supplements, foods, and health products represented a $1.4 billion market in 2018 and is forecasted to reach $10.3 billion by 2025 (Value Market Research, 2020). For this reason, cannabidiol oil, in addition to other cannabinoids, are expected to be released into water systems and the environment (How and Gamal El-Din, 2021), raising environmental concerns as a growing body of work is showing that households chemicals, pharmaceuticals, and illicit drugs can accumulate in waterways and impact on aquatic life (Richmond et al., 2017; Rosi-Marshall et al., 2015). For example, THC metabolites are rapidly accumulating in different environments such as surface water, ground water, and wastewater effluents (Boleda et al., 2009; Carmona et al., 2014; Mackuľak et al., 2014; Mastroianni et al., 2016). Because cannabidiol has a similar structure to THC and the compound is known to be relatively stable, at least under laboratory

conditions (Mazzetti et al., 2020), cannabidiol is also predicted to accumulate in the environment. Indeed, the rare studies considering cannabidiol detected the molecule in a high proportion of sludge samples (Mastroianni et al., 2013) and ground water samples tested (Mastroianni et al., 2016), and at concentration averaging 3.5 ng g^{-1} and 3.3 ng L^{-1}, respectively. Cannabinoids thus represent an important group of emerging pollutants with possible impact on aquatic organisms.

Cannabidiol could impact aquatic organisms in at least two ways. First, cannabidiol can interact with the endocannabinoid system of all vertebrates (Elphick, 2012). While the role of the endocannabinoid system is not fully understood, cannabinoids can be absorbed through the skin of fishes and interact with cannabinoid receptors (Krug and Clark, 2015). Indeed, recent experimental work conducted in zebrafish showed that embryos exposed to the higher end of a therapeutically relevant concentration of cannabidiol could harbor multigenerational developmental neurotoxicity effects most likely due to changes in gene expression (Carty et al., 2019, 2018). On the other hand, another study showed that zebrafish exposed to similar cannabidiol concentrations during early development (i.e., 6 h post-fertilization) presented increased survival to adulthood and longevity (Pandelides et al., 2020).

Given the fact that the zebrafish microbiome is highly influenced by environmental factors (Roeselers et al., 2011; Stephens et al., 2016), cannabidiol could also impact aquatic organisms healthy development by altering the animal's microbiome. Disruption of intestinal microbiomes, known as dysbiosis, due to environmental pollutants have been found to lead to developmental issues in at least four species of fishes (Austin and Al-Zahrani, 1988; Narrowe et al., 2015; Schmidt et al., 2016; Zhou et al., 2018) and even early mortality in zebrafish larvae (Pindling et al., 2018a, 2018b). While no previous work studied the impact of cannabidiol on fish microbiomes, the molecule was shown to exhibit antimicrobial activity against certain Gram-positive bacteria such as *Staphylococcus aureus* (Van Klingeren and Ten Ham, 1976; Wassmann et al., 2020) and to reduce gut inflammation in patients with obesity (Al-Ghezi et al., 2019; Cluny et al., 2015; Mestre et al., 2018), suggesting that the molecule can indeed affect microbial life. While it is believed that cannabidiol oil has no negative effect on the human microbiome (Di Marzo and Izzo, 2006; Kalaydina et al., n.d.; Mestre et al., 2018), it is essential to consider the possible impacts of cannabidiol on more susceptible aquatic organisms, especially at life stages where the microbiome is still developing.

Here, we investigate the potential impact of passive exposure to cannabidiol oil on healthy zebrafish larvae's microbiome. Zebrafish are a well-established model for gut health (Brugman, 2016) and environmental toxicology given their high sensitivity to possible contaminants (Ali, van Mil and Richardson 2011), providing an ideal test ground for the possible unforeseen effects of cannabidiol on animal hosts. Based on previous work, we exposed the zebrafish larvae to therapeutically relevant cannabidiol oil concentrations expected to exceed environmental concentrations (Valim Brigante et al., 2018). Given the evidences that cannabidiol can affect microbial growth and zebrafish larvae's microbiome sensitivity to pollutant exposure, we expected to observe significant changes in the animal's microbiome and a possible detrimental effect on its survival.

2. Materials and methods

2.1. Zebrafish husbandry

Zebrafish (*Danio rerio*) were maintained in the Biology Department of Bard College laboratories following standard protocols for zebrafish husbandry (Krug and Clark, 2015). Experimental populations of zebrafish strain Et20 were raised in a 14-h light:10-h dark cycle in standard recirculating rack water kept at 28.5 °C with pH ranging from 7.0 to 7.4. Furthermore, this study was approved by the Bard Institutional Animal Care and Use Committee before proceeding (IACUC; most recent approval I.D. "Perron, 2018″).

2.2. CBD oil quality

We selected the CBDPure hemp oil 100 (Full Spectrum CBD oil, Nutra Pure LLC, Vancouver, WA) based on publicly available product quality reports. Indeed, the CBD oil extract used in this study was certified by a third-party for cannabinoid profiling, pesticide testing, microbiological screening, terpene analysis, and residual solvent. In addition to third-party certification from the manufacturer, we validate the CBD oil quality internally using gas chromatography/mass spectrometry analysis. More specifically, we extracted cannabinoids using methanol (MeOH, Sigma-Aldrich, HPLC grade) without further purification. Extractions of commercial CBDPure 100 hemp oil (1.00 g samples, 12 × 1 mL MeOH) were performed in triplicate, combined, concentrated in vacuo, and redissolved in 1 mL MeOH before GC/MS analysis. Cannabinoid analytical standards: cannabidiol solution (CBD, 1.0 mg/mL in methanol, certified reference material), cannabinol solution (CBN, 1.0 mg/mL in methanol, certified reference material), and Δ^9-tetrahydrocannabinol solution (THC, 1.0 mg/mL in methanol, certified reference material) were purchased from Sigma-Aldrich. A calibration curve for the CBD solution standard was obtained in triplicate and was linear (R^2 > 0.992) over the measured concentration range (0.03 mg mL^{-1} – 1 mg mL^{-1}).

We performed gas chromatography/mass spectrometry analysis using a Varian-450 GC equipped with a Varian FactorFour™ VF-5 (30 m x 0.25 mm, 0.25 μm) capillary column and a Varian-220 MS. Helium gas was used as the carrier gas in constant flow mode at 1 mL min^{-1}, and the solvent delay was set at 3 min. The injector was maintained in a 1:50 split ratio mode at 220 °C while the oven was programmed as follows: 100 °C (2 min) to 260 °C at a heating rate of 15 °C min^{-1} (5 min). The G. C. oven program's run time was 20 min, and the mass spectrometer was in full scan mode in the range of *m/z* 60–350. Of the standards analyzed, we detected only the cannabinoid CBD at a concentration of 0.56 ± 0.07 mg mL^{-1} (Figure S1). Finally, the M.S. provides conclusive evidence that a large concentration of CBD was indeed detected in CBDPure 100 (Figure S2). While the concentration of CBD detected with our methodology is lower than that reported by NutraPure, the analyses provided by a certified lab did indeed report 1.6 mg mL^{-1}. We will hereafter use the concentration reported by the certified third-party analytical laboratory.

2.3. Experimental populations

For each independent trial, between 20 and 30 fertilized eggs were sampled approximately one hour after a single mating and bleached twice in 0.5% hypochlorite solution for four minutes and once in sterile 1x E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$, 0.5 mg/L methylene blue). Fertilized eggs were placed in sterile Petri dishes filled with 60 mL of 1x E3 medium, of which 80% was replaced with fresh medium every 24 h and monitored for possible contaminants and embryo mortality.

For each trial, we established two control populations and two experimental populations using 24 healthy larvae (i.e., 5-day-post-fertilization (dpf)) for a total of 96 animals. Each larva, randomly selected from the larger pool, was placed in a single well of a 24-well plate filled with 2 mL of sterile 1X E3 medium. One control population, referred to as the control group, was placed in sterile E3 medium. The second control group, referred to as the DMSO group, was exposed to 0.05% DMSO, the concentration of DMSO used as a solvent for cannabidiol oil. As for the experimental populations, one was placed in sterile E3 medium supplemented with 20 μg/L cannabidiol oil, and another was exposed to sterile E3 medium supplemented with 200 μg/L cannabidiol oil. The two concentrations were chosen to represent a range of toxicity for cannabidiol previously investigated in zebrafish larvae (Valim Brigante et al., 2018). Cannabidiol solution was made fresh before each independent trial using a commercially available CBD oil (Full Spectrum CBD oil, Nutra Pure LLC, Vancouver, WA) and dissolved in a 5% DMSO

solution. While most cannabinoids are known to be adsorbed to plastics over time, recent work showed that plastic tubes and glass tubes did not shown significant difference in adsorption/degradation in laboratory condition similar to the one described above (Bruno et al., 2019) or at concentration above the one used in this study (Molnar et al., 2013). Furthermore, approximately 80% of the medium was exchanged daily for sterile 1x E3 media with the relevant cannabidiol or DMSO concentration when applicable to minimize the risk of contamination and to maintain the cannabidiol oil concentration as close as possible to the original concentrations.

We maintained the zebrafish larvae in experimental conditions for five days without feeding and assessed survival daily. Based on our previous work (Dahan et al., 2018; Pindling et al., 2018a), this timeline is sufficient for the host-microbiome to develop to a size fit for investigation without affecting survival. At the end of the experiment, three larvae from each treatment, for a total of 12 animals, were randomly selected and anesthetized according to established euthanasia techniques (Matthews and Varga, 2012; NIH, 2009). Following euthanasia, fish were washed three times with nuclease-free water to minimize the presence of free-living bacteria and stored at −80 °C. The procedure was repeated over three trials using independent broods from the same mating pair for a total of 36 preserved animals (i.e., 12 animals per trial).

2.4. DNA extraction and processing

We extracted and purified microbial DNA from each fish larvae using a modified protocol for the DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD) as implemented in (Pindling et al., 2018b, 2018a). Purified gDNA samples were stored in nuclease-free H_2O at −20 °C. Fish microbiomes were characterized via targeted gene amplification of the 16S rRNA V4 region using Golay-barcoded primers 515F and 806R (Caporaso et al. 2012). PCR and sequencing reactions were performed at the Wright Labs (Huntingdon, PA) using the MiSeq paired-end Illumina platform adapted for 250-bp paired-end reads as previously described in (Dahan et al., 2018; Pindling et al., 2018a). All sequence reads are available at the Sequence Read Archive of the NCBI (PRJNA692159).

2.5. Processing of 16S rRNA sequence data

Microbiomes were characterized using the DADA2 pipeline version 1.8.1 (Callahan et al. 2016) available at (https://github.com/benjjneb/dada2) and implemented in R version 3.2.3 (http://www.r-project.org). In brief, forward and reverse reads were filtered, de-replicated, and de-noised using *DADA2's* default parameters. After removing chimeras and all sequences identified as either mitochondrial or chloroplast DNA, each unique sequence is defined as an amplicon sequence variant (or ASV). A full list of ASV and their abundance in each sample can be found in Table S1. For each ASV, we then assigned taxonomy using *IDTAXA* (Murali et al., 2018) available via the *DECIPHER* Bioconductor package (DOI: 10.18129/B9.bioc.DECIPHER) trained on the SILVA ribosomal RNA gene database version 138 (Quast et al., 2013) as well as the RDP trainset 16 (McDonald et al., 2012; Werner et al., 2012). Full taxonomic assignment, along with DNA sequences, can be found in Table S2. Lastly, we build a maximum likelihood phylogenetic tree based on a multiple alignment of all the ASVs using the *phangorn* package version 2.1.3 (Schliep 2011).

2.6. Data visualization and statistical analyses of 16S rRNA sequence data

Patterns of diversity within the ASV tables were analyzed using a modified version of the pipeline described in Dahan et al. (2018), implemented in *R* and visualized in *ggplot2* (Wickham, 2009). A mapping file linking sample names and the different treatments is provided in Table S3. Briefly, we used *phyloseq* version 1.30.0 (available at https://joey711.github.io/phyloseq/) to compare the relative

abundance of the most common (i.e., >15%) ASVs across treatments. For each sample, we estimated richness as the number of ASVs as well as Chao1 and Simpson (*D*) from the ASV table subsampled to the lowest sampling depth for any sample, i.e., 5007 paired-reads. We then tested whether richness and diversity indices differed among treatments using linear modeling and comparing the different statistical models with Akaike's Information Criterion (AIC) as implemented in *R's* stats package.

To identify possible differences in community composition between treatments (e.g., control microbiomes vs. microbiomes exposed to DMSO as well as control microbiomes vs. microbiomes exposed to cannabidiol), we performed Principal Coordinate Analyses (PCoA) on Morisita-Horn similarities estimated between each sample. We also performed PCoA on phylogenetic distances between samples calculated as weighted UniFrac distance scores (Lozupone and Knight 2005). Each test's significance was estimated using a permutational analysis of variance (PERMANOVA) using the *adonis* function (Oksanen et al., 2015) of *vegan* version 2.5.6. We tested for possible differences in community composition between each treatment using the *pairwise.adonis* function of the *pairwiseAdonis* package v0.3. To confirm the homogeneity of variances among and within treatments, we used the *vegan* package implementation of *PERMDISP2* via the betadisper method (Oksanen et al., 2015). Finally, we investigated possible changes in the abundance of individual ASVs between treatments using *DESeq2* differential abundance comparison (Love, Huber, and Anders 2014) adapted for use with microbial count data (McMurdie and Holmes, 2013).

3. Results

3.1. Larvae survival after exposure to CBD oil

We first investigated whether exposure to cannabidiol oil affected zebrafish larvae survival by monitoring larval death daily throughout the experiment. We found no significant difference in mortality between the different treatments in all three independent trials: Trial 1 ($\chi^2_{(3, N = 480)} = 2.2$; $P = 0.50$; Fig. 1A); Trial 2 ($\chi^2_{(3, N=)} = 0$; $P > 0.99$; Fig. 1B); and Trial 3 ($\chi^2_{(3, N = 480)} = 3.9$; $P = 0.30$; Fig. 1C). Interestingly, when combining the mortality events for all three trials, we found that the proportion of animals that survived throughout the study is significantly higher in populations exposed to cannabidiol oil (Fisher's Exact Test; $P = 0.03$). More specFisher's, we observed a total of eight death events among the control groups over the three trials, and we recorded only two deaths among the groups exposed to cannabidiol oil.

3.2. 16S rRNA V4 reads using DADA2

After removing non-desired sequences, we retained 1056,920 (or 90.0% of the total) sequence reads from 35 larval microbiomes, which resulted in 782 unique amplicon sequence variants (ASVs). Using 16S rRNA profiling, the ASVs were assigned to 17 phyla, 79 orders, and 192 known genera (see Table S2 for full listing). While 256 (or 32.7% of all) ASVs could not be identified at the genus level, the taxonomic assignment was consistent when using different reference databases (Figure S3). Further results will hereafter be presented for taxonomic profiling based on the SILVA database release 138.

To investigate the stability of the zebrafish larval microbiome in our study, we then compared the microbiomes of eight control larvae sampled randomly from the tree trials we conducted; one of the microbiomes from trial T1 failed to be sequenced. Among the eight microbiomes extracted from control larvae, we recovered all of the 782 ASVs sampled from our whole study, demonstrating the zebrafish larval microbiome variability. Comparable to previous work done of the core microbiome of zebrafish living in different environments (Roeselers et al., 2011), we found that a majority of ASV belonged predominantly to the phylum Proteobacteria, 80.2(5.0)%, and, to a lesser extend,
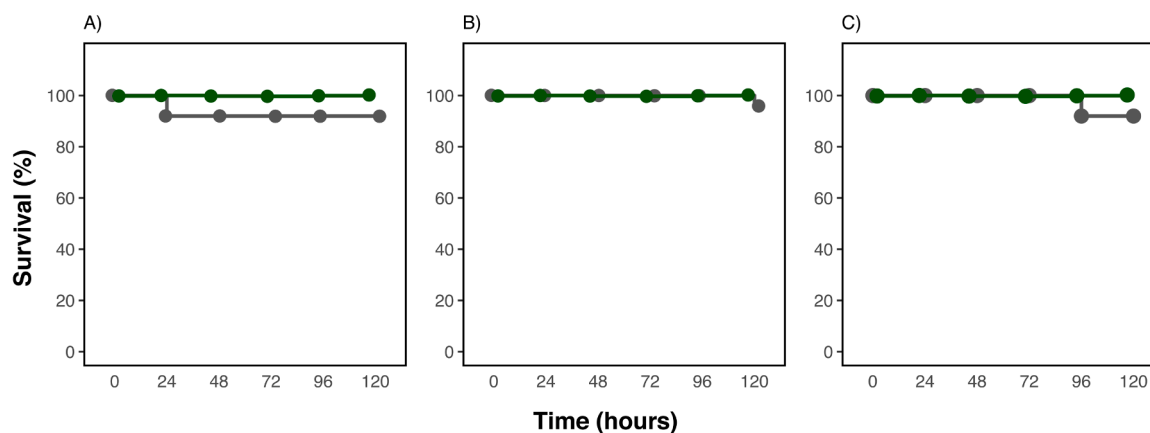
Fig. 1. Survival curves of zebrafish larvae exposed to cannabidiol oil. Survival was estimated daily in a population of 24 control fish, 24 fish exposed to DMSO (gray), 24 fish exposed to 20 µg mL^{-1} cannabidiol oil (not shown for illustration purposes), and 24 fish exposed to 200 µg mL^{-1} cannabidiol oil (green). We found no significant difference among the treatments in three independent trials: A) Trial 1 ($\chi^2_{(3, N = 480)} = 2.2$; $P = 0.50$); B) Trial 2 ($\chi^2_{(3, N = 480)} = 0$; $P > 0.99$); C) Trial 3 ($\chi^2_{(3, N = 480)} = 3.9$; $P = 0.30$). Survival between all control and experimental populations were compared using the Kaplan-Meier log-rank test.

Firmicutes, 9.6(0.1)%, (Fig. 1A). While some of the most common ASVs we identified as part of the *Pseudomonas* genus, totalizing up to 17.1 (0.07)% of the total abundance, a large number of ASVs, 39.8(0.05)%, could not be matched to a known genus. Also, while the composition of the different control microbiomes differed somewhat between samples, with 62.7% similarity as measured with the Morisita-Horn Similarity Index, we found no overall significant dissimilarity between the three independent trials ($F_{(2, 5)} = 2.69$; $R^2 = 0.52$; $P = 0.07$). Possible dissimilarities between control microbiomes were even less significant when considering phylogenetic distance within the communities measured as weighted UniFrac ($F_{(2, 5)} = 1.56$; $R^2 = 0.38$; $P = 0.14$), meaning that the ASVs that differed between trials were closely related genetically and predicted to fill similar ecological niches.

Lastly, we tested whether the presence of DMSO, the solvent used to dissolve cannabidiol in the growth medium, affected zebrafish larval microbiomes by comparing the microbiome of larvae exposed to DMSO at a concertation similar to that used in the main experiment to that of the control microbiomes. We found that DMSO did not significantly alter the zebrafish larval microbiomes ($F_{(1, 10)} = 1.08$; $R^2 = 0.06$; $P = 0.31$).

Once again, the effect was not significant when considering the phylogenetic distance among the communities ($F_{(1, 10)} = 1.03$; $R^2 = 0.07$; $P = 0.38$; Fig. 1B). Interestingly, when combining the control microbiomes to the microbiomes of larvae exposed to DMSO as a single control group, we found a small difference between the three trials when considering the similarity between the two control groups ($F_{(2, 5)} = 3.42$; $R^2 = 0.38$; $P = 0.033$) as well as the phylogenetic distance between the two control groups ($F_{(2, 5)} = 2.39$; $R^2 = 0.30$; $P = 0.03$). While this difference is not significant when applying a correction for multiple statistical testing, we will test the possible effect of trials on CBD treatment in subsequent analysis.

3.3. Cannabidiol does not affect the diversity of zebrafish larval microbiome

To investigate whether cannabidiol could impact zebrafish larval microbiome, we first compared the overall microbial diversity in microbiomes sampled from control larvae to that of microbiomes sampled from larvae exposed to either 20 µg/L or 200 µg/L. We found no
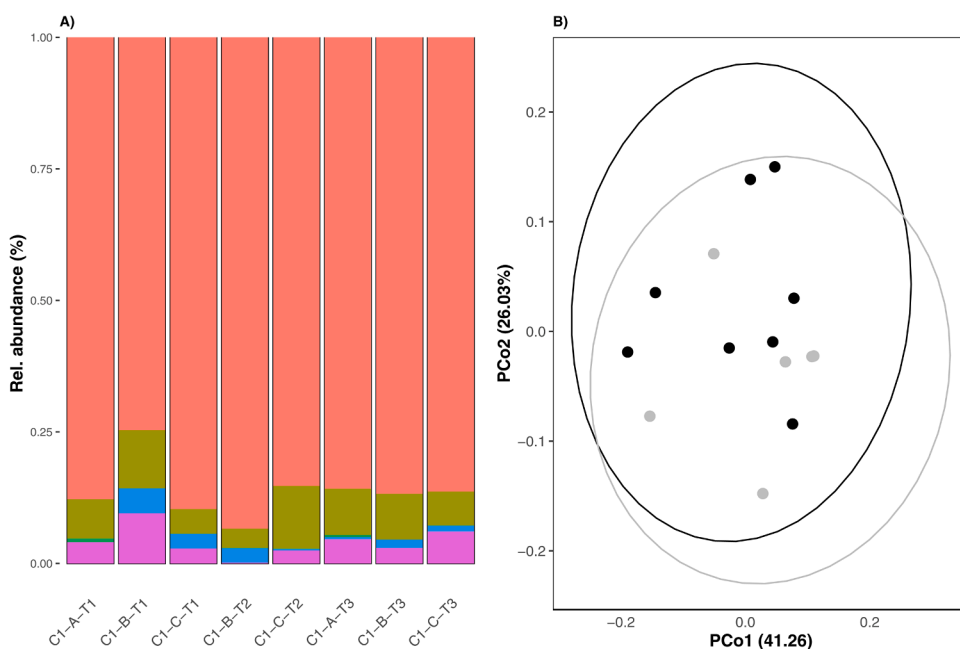

Fig. 2. Establishing the baseline microbiome of zebrafish larvae. A) While we found that the microbiomes of zebrafish larvae varied greatly at the sequence variants level, with 68% average similarity between the different microbiome sequenced, we found that the microbiomes of control zebrafish were stable over three independent trials ($F_{(2, 5)} = 1.56$; $R^2 = 0.38$; $P = 0.14$), showing similar relative abundance of phyla at seven days post-fertilization. For visualization purposes, sequence variants under 15% relative abundance were left out; Actinobacteriota is shown in fuchsia, Bacteroidota in blue, Deinococcota in green, Firmicutes in brown, and Proteobacteria in vermillion. **B)** Using phylogenetic distance, estimated as weighted UniFrac scores, to compare microbial communities, we found that DMSO (gray), used as a solvent for cannabidiol oil in this study, did not affect community composition compared to control populations (black)($F_{(1, 10)} = 1.03$; $R^2 = 0.07$; $P = 0.38$).

significant differences in average richness, estimated as the total number of ASVs, among the different groups ($F_{(2, 19)} = 1.26$; $P = 0.31$; Fig. 2A) and this was true in all three trials ($F_{(2, 5)} = 0.48$; $P = 0.63$). On average, we found a total of 53.86(7.7) ASVs in the control microbiomes, 56.86 (16.5) ASVS in the fish exposed to 20 µg/L, and 46.86(10.3) in the fish exposed to 200 µg/L. Interestingly, we found similar richness when considering the Chao1 index, which estimates the number of predicted taxa expected if the population was infinite (Table 1), indicating that our sampling was exhaustive.

We then look at measures of diversity that include evenness. The latter is important in defining community diversity as it indicates whether one or a few species dominate the community. Again, we found no significant difference between the three treatments estimated as Simpson's D Index ($F_{(2, 19)} = 0.56$; $P = 0.58$; Fig. 1A). On average, we found a Simpson Index of 0.87(0.08) in the control larvae microbiome, 0.84(0.15) in the 20 µg/L cannabidiol oil treatment, and 0.83(0.5) in the 200 µg/L cannabidiol oil treatment. These results indicate that, on average, two ASVs taken at random in any group have an ~85% chance of being different from each other, demonstrating that no ASVs tend to dominate the larval microbiome even in the presence of higher concentrations of cannabidiol.

3.4. Cannabidiol minimally alters the zebrafish larval microbiome structure

To further investigate whether cannabidiol could impact zebrafish larval microbiome, we compared the microbial community structure in microbiomes sampled from control larvae to microbiomes sampled from larvae exposed to cannabidiol oil. As opposed to microbial diversity, microbial community structure compares the identity of the different ASVs found in each group and their abundance. Here, we first compared the population structure between the different microbiomes using the Morisita-Horn Similarity Index, which quantifies the overlap between any two communities, and found that cannabidiol had a significant effect on community composition ($F_{(1, 19)} = 4.25$; $R^2 = 0.18$; $P = 0.002$; Fig. 3B). More specifically, we observed a difference in community structure between control fish microbiomes and microbiomes from fish exposed to 200 µg/L (pairwise-$F = 5.13$; adj-$P = 0.039$), with an average similarity of 66%. We found no statistical differences in community structures between the control microbiomes and the microbiomes exposed to 20 µg/L cannabidiol oil. We also found that the effect of cannabidiol oil differed somewhat across the different trials (interaction: $F_{(4, 19)} = 3.33$; $R^2 = 0.29$; $P = 0.001$). Mainly, we found that community structure did not differ significantly under the effect of cannabidiol oil in trial T3 ($F_{(2, 8)} = 1.17$; $R^2 = 0.23$; adj-$P = 0.90$), suggesting that the effect of cannabidiol is either weak statistically or not consistent across different trials.

We also investigate community structure changes based on phylogenetic distance, which accounts for possible predicted redundancy in ecological functions; closely related ASVs are predicted to carry similar functions. Here again, we found that cannabidiol had a significant effect on microbial structure ($F_{(2, 19)} = 2.74$; $R^2 = 0.15$; $P = 0.007$). Once again, we observed the main difference between fish microbiomes and microbiomes from fish exposed to 200 µg/L (pairwise-$F = 3.17$; adj-$P = 0.039$) and found no differences between control microbiomes and microbiomes exposed to 20 µg/L. Interestingly, the significant interaction between the effect of cannabidiol and the different trials (interaction: $F_{(4, 19)} = 2.47$; $R^2 = 0.27$; $P = 0.002$) was due to the fact that we

observed no effect of cannabidiol on phylogenetic structure in trial T1 ($F_{(2, 8)} = 0.026$; $R^2 = 0.026$; adj-$P = 0.60$) as opposed to trial T3 when considering community overlap above. Moreover, when considering phylogenetic distance, trial had no significant impact on community structure as a main effect ($F_{(2, 19)} = 1.56$; $R^2 = 0.08$; $P = 0.09$), suggesting a greater effect of phylogenetic redundancy between trials.

Finally, we found that the changes in community structure observed between control microbiomes and microbiomes of fish exposed to 200 µg/L were dominated by only a few ASVs (Fig. 3C). More specifically, we found that exposure to cannabidiol decreased the abundance of two ASVs identified as *Methylobacterium-Methylorubrum* sp. and one ASV identified as *Staphylococcus* sp., two genera that are usually not part of the zebrafish core microbiome. On the other hand, exposure to cannabidiol increased the frequency of one ASV identified as *Chryseobacterium* sp., a bacterium commensal to zebrafish.

4. Discussion

Due to its popularity as a food supplement, cannabidiol oil is now detected as a pollutant in different waterways (Mastroianni et al., 2016). Given the predicted increase in cannabidiol oil use (Value Market Research, 2020), it is crucial to understand the full range of possible effects of cannabidiol on aquatic animals. Here, we show that cannabidiol oil has a limited effect on the zebrafish larval microbiomes at concentrations previously shown to impact on the animal gene regulation and neurological development (Carty et al., 2018; Valim Brigante et al., 2018). In addition, we show that exposure to cannabidiol oil increases larval survival, confirming previous results obtained under similar experimental conditions (Pandelides et al., 2020).

Overall, we found that exposure to 20 and 200 ng/L cannabidiol oil, two concentrations that were previously described as clinically relevant (Valim Brigante et al., 2018), did not cause a significant change in overall microbial diversity or microbial population structure. Given the sensitivity of the zebrafish larval microbiome and its importance for the animal's development (Roeselers et al., 2011; Stephens et al., 2016), this is an important and surprising result. In comparison, we recently found that exposure to streptomycin and arsenic at concentrations below clinical relevance induced significant reductions in microbial diversity and significant changes in microbial population structure of the zebrafish microbiome (Dahan et al., 2018; Pindling et al., 2018a).

The limited effect of cannabidiol oil on the zebrafish microbiome in our study could be explained by the reduction of the molecule's activity due to environmental factors such as inhibitors. Yet, refreshing the cannabidiol oil-supplemented medium on a daily basis likely limited the activity of such inhibitors (Bruno et al., 2019; Molnar et al., 2013). Crucially, while we did not observe overall changes in microbial diversity or population structure in the zebrafish microbiomes, we detected a few significant changes at the bacterial variant level in animals exposed to cannabidiols oil when compared to control animals, confirming that the animals indeed absorbed the molecules.

More specifically, we found that exposure to 200 ng/L cannabidiol oil significantly reduced the abundance of three sequence variants. Two of the sequence variants were identified as *Methylobacterium* sp., a common environmental bacterium that generally harbors multiple copies of the *16S rRNA* gene and can infect immuno-compromised zebrafish (Dahan et al., 2018; Yang et al., 2017). Cannabidiol oil also reduced the abundance of one ASV identified as *Staphylococcus* sp., a gram-positive bacterium found to be susceptible to cannabidiol's antimicrobial activity (Van Klingeren and Ten Ham, 1976; Wassmann et al., 2020). Interestingly, all three bacterial variants are not associated with the zebrafish commensal microbiome (Roeselers et al., 2011; Valenzuela et al., 2018).

Conversely, cannabidiol oil exposure increased the abundance of one ASV identified as *Chryseobacterium* sp.. The latter is a gram-negative bacterium that is part of the zebrafish commensal microbiome and that is predicted to modulating the animal's immune system (Koch et al.,

Table 1
Mean(s.d) of alpha diversity metrics.

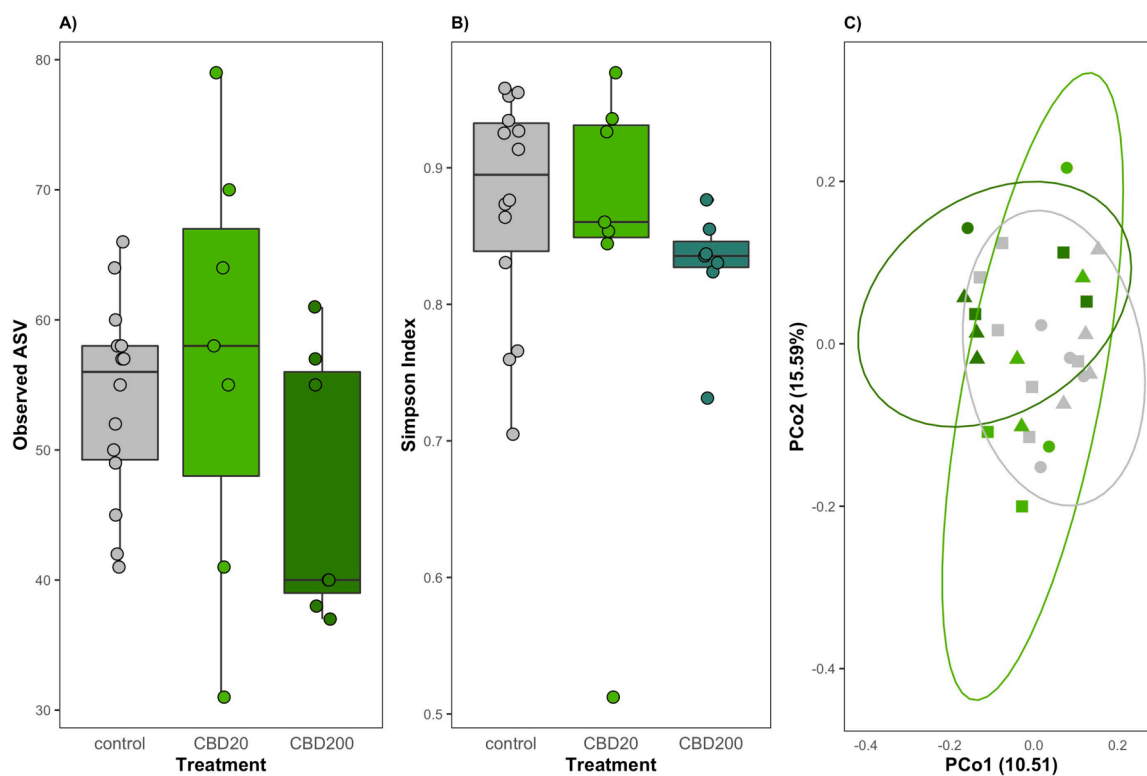| Treatment	Observed	Chao1	Simpson
Control	53.9(7.7)	54.0(7.8)	0.87(0.08)
20 µg/mL	56.9(16.5)	57.8(16.2)	0.84(0.15)
200 µg/mL	46.9(10.3)	47.8(10.3)	0.83(0.05)

Fig. 3. The effect of cannabidiol on zebrafish larval microbiome. We found that the microbiomes of zebrafish larvae exposed to 20 µg/L (pale green) or 200 µg/L (dark green) of cannabidiol did not affect **A**) the total number of ASVs, i.e., richness ($F_{(2, 19)} = 1.26$; $P = 0.31$) or **B**) diversity measured as Simpson's D ($F_{(2, 19)} = 0.56$; $P = 0.58$) when compared to the microbiomes of control zebrafish at seven days post-fertilization. This effect was consistent over three independent trials. **C**) Using phylogenetic distance, measured as weighted UniFrac scores, we found that microbiomes exposed to 200 µg/L had a small differences when compared to control microbiomes ($F_{(2, 19)} = 2.74$; $R^2 = 0.15$; $P = 0.007$).

2018; Murdoch and Rawls, 2019) as well as protecting the fish against pathogens following disturbance due to antibiotic exposure (Stressmann et al., 2020). Because the zebrafish larval microbiome is heavily influenced by microorganisms present in the environment (Roeselers et al., 2011), whether cannabidiol affected microbial communities directly within the fish or via selective pressures imposed on the environment remains to be tested. Additional studies are also required to understand the direct mechanisms in which cannabidiol increases or decreases the abundance of specific taxa and whether such changes are sustained at different life stages or after exposure is stopped.

Finally, we also found that exposure to cannabidiol oil increased zebrafish larvae survival over the duration of the experiment. While the number of deaths observed in the control populations was comparable to other similar studies (Dahan et al., 2018; Pindling et al., 2018a), we consistently observed fewer mortality events in the larvae population exposed to cannabidiol oil. Interestingly, small increases in survival have been documented under the effect of moderate environmental stresses, a phenomenon known as the hormetic effect (Choi et al., 2012). While further work will be necessary to elucidate the mechanism responsible for this finding, cannabidiol was recently observed to prolong longevity in adult zebrafish also exposed at the larval stage (Pandelides et al., 2020).

5. Conclusion

Taken together, our results suggest that cannabidiol oil is unlikely to have a significant impact on fish larvae microbiomes at an early developmental stage. Yet, as the use of cannabidiol oil keeps rising, further study will be required to investigate the possible effects of the molecule on aquatic life at different life stages. Furthermore, while this study focused on cannabidiol oil, the production of cannabidiol oil

generates many by-products that could also affect aquatic organisms in different ways (Pavlovic et al., 2018). Furthermore, the long-term effects of exposure to cannabidiol oil concentrations likely to be detected in the environment remain hard to predict (Boleda et al., 2009). For these reasons, we believe that it is important to establish monitoring programs to keep track of cannabidiol oil in the environment.

CRediT authorship contribution statement

Maracela Talamantes: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Stella Rose Schneeberg:** Investigation. **Atahualpa Pinto:** Conceptualization, Investigation, Supervision, Writing – original draft. **Gabriel G. Perron:** Conceptualization, Formal analysis, Supervision, Writing – review & editing, Visualization.

supplemented medium.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2021.100045.

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