THE IMPACT OF CYANOTOXIN EXPOSURE ON THE MICE GUT MICROBIOME COMMUNITIES STRUCTURE

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Evance Pakuwal

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Thesis written by

Evance Pakuwal

B. Sc, Tribhuvan University, 2016

M.S., Tribhuvan University, 2020

M.S., Kent State University, 2023

Approved by

Xiaozhen Mou, Ph.D. _____, Advisor

Heather Caldwell, Ph.D. _____, Chair, Department of Biological Sciences

Mandy Munro-Stasiuk, Ph.D. _____, Dean, College of Arts and Sciences

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CHAPTER I

GENERAL INTRODUCTION

Access to clean drinking water is a cornerstone of robust public health infrastructure. Yet, this fundamental necessity is increasingly threatened due to the escalating incidence of cyanobacterial harmful algal blooms (CyanoHABs) in freshwater environments. These blooms, which have become a global concern (Paerl & Huisman, 2009; Paerl & Otten, 2013), are primarily driven by eutrophication, which is a consequence of intensified agricultural and industrial activities, coupled with inadequate water management practices and burgeoning human populations (Anderson et al. 2015).

CyanoHABs are capable of producing cyanotoxins and forming dense, visible blooms in both freshwater and marine environments (Zanchett & Oliveira-Filho, 2013). The proliferation of CyanoHABs is influenced by a complex interplay of factors, including temperature, nutrient levels, and water quality (Carmichael et al. 2016; Paerl & Huisman, 2009). The specific contribution of these factors can vary depending on the cyanobacterial species involved (Gilbert et al. 2005). Additional elements such as light intensity, salinity, and the presence of other microorganisms also play a role in CyanoHAB formation (Paerl et al.2001).

CyanoHABs have become a significant disruptor in freshwater ecosystems worldwide, and their incidence and severity have increased over time (Figueiredo et al. 2004; Oliver & Ganf, 2000; Paerl et al. 2016). Similar patterns have been noted in Asian nations, including China (Liu et al. 2017) , Japan (Kurobe et al. 2016), and South Korea, as well as European countries like Finland

and Germany (Kim et al. 2017; Pelaez et al. 2010).

Due to the worsening eutrophication and land-use changes brought on by the rising worldwide need for food and energy, CyanoHAB frequency and severity are predicted to remain high (Khan & Mohammad, 2014).

Land use practices can impact CyanoHABs in multiple ways, such as nutrient pollution from agricultural runoff and urban stormwater, changes in water temperature caused by climate change, and alterations in water flow due to dam construction or water diversion (Álvarez et al. 2017). Considering the potential impacts of CyanoHABs on water quality, practices can be implemented to reduce nutrient pollution, mitigate climate change, and minimize alterations in water flow (Cheng et al. 2022).

One important negative impact of CyanoHABs is the production of cyanotoxins, which can have significant consequences on both human and animal health as well as the ecological balance of aquatic ecosystems (Brooks et al. 2016).

Diversity of cyanotoxins

A diverse array of potent cyanotoxins can be produced during CyanoHABs; the common examples are microcystins (MCs), saxitoxins, and anatoxins (Papadimitriou et al. 2010).

MCs, primarily produced by *Microcystis*, *Anabaena,* and *Planktothrix*, are cyclic peptides with a unique amino acid, ADDA, crucial for their toxic activity (Catherine et al. 2016). MCs cause cell death by excessively phosphorylating proteins, which they do by predominantly attacking the liver (Shi et al. 2021). By inhibiting protein phosphatases, particularly PP1 and PP2A, MCs disrupt the balance of protein phosphorylation and dephosphorylation, leading to cellular dysregulation and apoptosis (Derminio, 2020).

There are over 100 variants of MCs, to name a few Microcystin-Arginine-Arginine (MC-RR), Microcystin-Tyrosine-Arginine (MC-YR), Microcystin-Leucine-Arginine (MC-LR), and Microcystin-Lysine-Arginine (MC-LA), they exhibit varying degrees of toxicity, (Díez-Quijada et al. 2019; Vesterkvist et al. 2012), which is dependent on factors such as chemical structure, concentration, exposure duration, and route of exposure (Díez-Quijada et al. 2019; Vesterkvist et al. 2012).

Saxitoxins are produced by some marine dinoflagellates and by freshwater cyanobacteria such as *Aphanizomenon, Dolichospermum*, and *Cylindrospermopsis* (USEPA, 2019; WHO, 1998). Saxitoxins is a type of neurotoxins and primarily act by blocking voltage-gated sodium channels, which can result in paralysis and death if present in sufficiently high concentration (Carmichael, 2001). Other neuronal channels, such as potassium and calcium channels, may also be affected by saxitoxins (Carmichael, 2001).

Anatoxins are produced by freshwater cyanobacteria, including *Anabaena* spp. (EPA, 2015: Sivonen & Jones 1999). Anatoxins primarily target the nervous system, leading to neurotoxicity and muscular paralysis (Harada et al. 2009; Osswald et al. 2007; Testai et al. 2016).

Health Concerns of Microcystins

Recent research indicates that MC-contaminated food or water may have adverse effects on organ systems, such as the kidneys and gastrointestinal tract, despite the fact that the hepatotoxic effects of MCs are well-documented (Chen et al. 2005; Stewart et al. 2016). However, the mechanisms and magnitude of impacts are not entirely understood, highlighting another crucial subject for further research.

MCs produced by the CyanoHABs are widely distributed in affected surface waters during the warm seasons; they cause ecological disruptions such as fish kills, wildlife poisoning, and

imbalances in aquatic ecosystems' food webs and nutrient cycles (Fischer et al. 2006; Harada et al. 2009). These toxins can eventually end up in the human system through various exposure routes, including consumption of contaminated water or food, aerosol inhalation, and skin contact during water-based activities (Melaram et al. 2022). Moreover, MCs can bioaccumulate in the food chain (Ding et al. 2021; Ni & Luo 2017; Zhang et al. 2021) and being absorbed in the agricultural crops through irrigation, posing a significant risk to human health (Malbrouck & Kesement, 2006; Melaram et al. 2022).

Specific public health concerns associated with exposure to cyanotoxins are an area of active research and public health concern. A drinking water guideline value of 1.6 μg/L has been adopted for people aged 6 years and older in the state of Ohio, USA by the Ohio Environmental Protection Agency (Farrer et al. 2015). The daily intake tolerable amount of MC has been set at 0.04 mg/kg body weight per day in the case of seafood (Arman & Clarke, 2021).

Despite the monitoring and mitigation strategies, major incidents related to exceeding MC concentrations take place very often. For instance, Lake Erie has routinely exceeded 20μg/L in the past and is only moderately safe for recreational use (Charmichael et al. 2017; World Health Organization, 2011). MC concentrations exceeded 1000 μg/L at Grant Lake St. Marys, Ohio's largest reservoir, an important recreational ground and drinking water source for residents in Celina, Ohio (Dumouchelle & Stelzer, 2014). The 2014 Toledo water crisis, caused by high levels of MCs in the city's water supply, underscored the public health implications of cyanotoxin contamination (Green, 2016). This incident highlighted the urgent need for a deeper understanding of the broader health impacts of MC exposure. Conventional water treatment facilities could be unable to completely remove MCs, which would result in their presence in final drinking water globally (Mohamed et al. 2015).

Gut Microbiome and potential disturbance from MC exposure

Gut microbiome, a diverse community of microorganisms inhabiting the gastrointestinal tract, is a crucial component of human health (Hills et al., 2019). With trillions of bacteria contributing to these processes, it is involved in vital processes like digestion, immunological control, and nutrition synthesis (Garcia-Montero et al. 2021). This intricate ecosystem, composed of bacteria, archaea, viruses, and fungi, is also instrumental in vitamin production, immune system modulation, and protection against pathogens (Heintz-Buschart et al. 2018).

Emerging research suggests that MC exposure can alter gut microbiome community structure (Lee et al. 2020), with potential implications for human health (Lone et al. 2015). For instance, a long-term study that compared the microbial communities in MC-LR exposed mice revealed a reduction in the relative abundance of bacterial taxa like *Actinobacteria, Saccharibacteria,* and *Prevotella*, which are known for their role in gut homeostasis (Zhuang et al. 2021). However, these studies have focused on overall outcomes and overlooked the potential dynamic during the length of exposure.

This dissertation delved into the impact of MC-contaminated water on gut microbiome community dynamics, a critical interaction that has yet to be fully understood. The findings of this study could offer novel insights on how MC exposure could go beyond liver toxicity and how the gut microbiome composition and diversity change over time in response to MC exposures (Sivonen & Jones, 1999). This study is particularly timely and relevant given the increasing prevalence of CyanoHABs and the potential health risks associated with cyanotoxin exposure.

Acute exposure (short-term and high intensity) to MCs can cause a range of health issues, from gastrointestinal symptoms to liver failure, but not necessarily disturb the gut microbiome (Wu et al. 2018). In a study that treated mice with 3000 μg/kg body weight (bw) and 4000-5000

μg/kg bw per day, the structure and diversity of gut microbiome did not change significantly over the course of a six-day experiment (Mills et al. 2021). This suggests that the gut microbiome may possess some resilience or adaptive mechanisms to withstand MC exposure.

Chronic exposure to low concentrations of MC-LR, has been linked to liver damage and other health issues due to the uptake of MCs by organs expressing organic anion transporter peptides (OATPs), similar to the impact of acute exposure (Blaha et al. 2009; Krishnan & Mou, 2021; Poste et al. 2011; Woolbright et al. 2017). However, contrasting results were found in the gut microbiome. A study by Lee et al. (2020) examined four groups of mice that are subjected to exposure of MC (water with 10 μg/kg MC-LR), lysate (water with *M. aeruginosa* extract normalized to 10 μg/kg total MCs), a negative control, and a positive control (500 mg/L phenobarbital, a liver tumor promoter). Their study lasted for 36 weeks, used the intraperitoneal route of exposure, and showed that the treated groups had similar gut microbiome communities as the positive control with tumor, which had significantly lower microbial diversity compared to the negative controls.

Two important bacterial phyla, *Firmicutes* and *Bacteroidetes*, which together account for over 90% of all bacterial abundance in the gut microbiome, are predominant in healthy gut of humans and their balance denotes the maintenance of gut homeostasis (Rinninella et al. 2019). The remaining 10% of the gut microbiome population is primarily made up of *Actinobacteria* and *Proteobacteria* (Arumugam et al. 2011; Segata et al. 2012). Probiotic bacteria like *Lactobacillus* and *Bifidobacterium* are another important sign of healthy gut microbiota (Dethlefsen et al. 2008; Gu et al. 2020; leBlanc et al. 2008; Qin et al. 2010; Zhu et al. 2021). Disruptions or imbalances in these taxa or microbial communities, known as dysbiosis, can lead to chronic inflammation and a range of health conditions, including inflammatory bowel disease, obesity, and metabolic disorders

(DeGruttela et al. 2016). Dysbiosis can also negatively impact the immune system, metabolism, and digestion, leading to inflammation in various organs and a weakened immune system (Carding et al. 2015, Yamashiro et al. 2017).

Although research on cyanotoxins and their effects has accumulated, potential gradual shifts in the structure of the microbial community during exposure at different concentration levels remain largely unexplored (Weng et al. 2007; Woolbright et al. 2017). This study aims to investigate the temporal and dose-dependent responses of the gut microbiome to MC-LR exposure, with a focus on identifying specific taxonomic shifts in both short and long terms.

General Objectives and Hypotheses

The general objective of this thesis was to understand the potential dynamic of changes in the gut microbiome of mice during MC-LR exposure via drinking. This investigation was guided by two primary hypotheses, each corresponding to a different plan of exposure duration and dosages of MC-LR.

Hypothesis 1: Short-term (weeks) exposure to low concentrations of MCs would trigger notable shifts in the gut microbiome. We anticipated that these modifications could result in gut dysbiosis, which might be an indication of a metabolic change in the mouse gut microbial communities.

Hypothesis 2: Long-term exposure (months) to low concentrations of MCs would cause significant alterations in microbial community structure. However, we predicted that the changes would be gradual but persistent. This could be a consequence of enduring selective pressures or slow adaptive responses within the microbial community.

Thesis Outline

Chapter I: ***General Introduction***. This chapter lays the foundation for the thesis by introducing cyanobacterial species, cyanotoxins, and CyanoHABs. It outlines the environmental and health issues associated with the use of MC-contaminated drinking water. The chapter concludes by identifying the research gaps that this thesis aims to address and presenting the research objectives and hypotheses.

Chapter II: ***The Impact of Short-term Exposure to Sub-lethal Dosage of Microcystins on Mice Gut Microbiome***. This chapter explores the impact of short-term exposure to MC-LR on the gut microbiome of mice. The hypothesis that short-term exposure of MC-LR leads to microbiome dysbiosis was tested and discussed.

Mice were subjected to doses of 50 μg/kg and 500 μg/kg MC-LR every other day via intragastric administration for 21 days. Fecal microbiome samples were collected in day 0, 7, 14 and 21 (4 time-points) and analyzed using Miseq Illumina 16S rRNA gene sequencing. Statistical analyses of the data were performed to explore potential correlations between MC-LR dosage and changes in microbiome composition.

Chapter III: ***The Impact of Long-term Exposure to Low Dosages of Microcystin on Mice Gut Microbiome***. This chapter investigates the impact of long-term exposure to MC-LR on the gut microbiome of mice. The mice were exposed to doses of 10 μg/kg and 50 μg/kg MC-LR every other day for 125 days. Similar to Chapter II, fecal samples were collected and analyzed, and statistical analyses were performed to identify potential correlations between MC-LR dosage and microbiome composition changes. The hypothesis that long-term exposure leads to microbiome dysbiosis, and that the changes will be more persistent is examined and discussed.

Chapter IV: *Summary*. The final chapter synthesizes the findings of the study, providing a comprehensive summary of the research. It concludes by discussing the implications of the research findings and suggesting potential avenues for future research in this field.

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CHAPTER II

THE IMPACT OF SHORT-TERM EXPOSURE TO SUB-LETHAL DOSAGE OF MICROCYSTINS ON MICE GUT MICROBIOME

INTRODUCTION

Cyanobacteria, pivotal primary producers within aquatic ecosystems, play vital roles in nutrient cycling, nitrogen fixation, and carbon sequestration (Ohkouchi et al. 2006). However, their biosynthesis of microcystins (MCs), pervasive waterborne pollutants, poses substantial hazards to human and animal well-being (Zanchett and Oliveira, 2013). MCs are cyclic peptides, with microcystin-leucine-arginine (MC-LR) being the most notorious due to their widespread presence and potent toxicity (Gupta et al. 2003). Understanding their interaction with living organisms is crucial.

CyanoHABs, widespread in global freshwater ecosystems, are significant sources of MC-LR. Exposure to MCs typically occurs from ingesting contaminated water or food, inhalation near affected water bodies, or dermal contact during recreational activities (Falconer & Humpage, 2005). Considering the pervasiveness of these common exposure routes, understanding the potential health implications of MCs is vital. The interaction of MC-LR with the gut microbiome is an emerging field, awaiting detailed exploration. The gut microbiome is a composite ecosystem within the gastrointestinal tract.

Gut microbiome plays essential roles in processes such as digestion, nutrient metabolism, and immune system development (Valdes et al. 2018). It also functions as a critical line of defense against harmful organisms (Lozupone et al. 2012). A healthy and diverse gut microbiome is central to maintaining host health, given its pivotal influence on both metabolic and immune responses (Wang et al. 2020). Although the gut microbiome is marked by its relative constancy, it can significantly respond to environmental toxins, such as MC-LR, with marked alterations observable within a matter of days (Uhr et al. 2019). Sarkar et al. (2019) used wild-type and gene knockout mice over two weeks and observed notable gut-related inflammatory changes post MC exposure, however, it was unclear when the response was triggered and if there were patterns. In contrast, Mills et al. (2021) explored the effects of a daily dose of MC-LR on gut microbiome of mice at 5000 μg/kg and 4000 μg/kg over a seven-day period and found no significant changes in the gut microbiome structure.

Mice have emerged as a reliable model for investigating MC-LR toxicity due to their close physiological and genetic alignment with humans (Mrdjen et al. 2018; Yan et al. 2023). Their gut microbiome bears a close resemblance to the human version, making them an ideal model organism. Additionally, their shorter lifespan allows for efficient studies on the long-term exposure effects of MC-LR (Yan et al. 2023).

We hypothesized that short-term exposures (weeks) to MC-LR at low concentrations would result in significant alterations to the composition of the gut microbiome, thereby triggering a state of gut dysbiosis. Dysbiosis describes a state of imbalance in the gut microbiota, which can be a decrease in the diversity of the microbiota, a loss of beneficial microbiota, or an overgrowth of harmful microbiota (Hrncir, 2022); this can have negative consequences for health. However, not all changes in the gut microbiome can be classified as dysbiosis, as the microbiome can

naturally fluctuate over time (Brüssow, 2020). To test this hypothesis, randomly grouped adult mice were administered MC-LR intragastrically, fecal samples were collected every week, and community DNAs were extracted. Then, the 16S rRNA gene sequencing of gut microbiomes was performed and the data received was analyzed. The findings enhanced our understanding of the health implications related to short-term MC-LR exposure.

METHODS

Experimental Setup

A total of 18 six-weeks-old male mice (C578B16) within the weight range of 25-30 g were received from Jackson laboratory-East (Bar Harbor, ME, USA), and they were acclimatized to the laboratory environment and human handling for 7 days in the animal housing located at the Kent State University animal facility before starting the experiment. For the acclimation, the animals were subjected to human handling and using feeding tubes with water and weighing every single day. Two or three mice of the same treatment group were housed together, and all the cages were environmentally controlled according to Institutional Animal Care and Use Committee (IACUC) guidelines.

All the animals had free access to a standardized chow diet and normal filtered water (ad libitum). The water was refreshed every two days.

After acclimatization, individual mice were randomly assigned into 3 different groups based on the dosage of MC-LR (0 µg/kg bw, 50 µg/kg bw, and 500 µg/kg bw) being provided via oral administration. These dosages were based on previous studies that have shown that MC-LR at these levels can cause liver toxicity and other health effects in mice and humans (Li et al. 2019). The median lethal dose (LD50) of orally administered MC-LR is 10.9 mg/kg in mice (Yoshida et al. 1997).

The animals were handled every other day by weighing them and by orally gavaging them the feeding tubes with the required toxins for the treatment mice and water for the control group. After each gavaging, each mouse was stimulated to allow releasing of the fresh fecal samples, and these samples were collected on Day 0, Day 7, Day 14, and Day 21 in sterile Eppendorf tubes and stored at -80°C immediately after collection until further processed.

DNA Extraction and 16S rRNA Gene Sequencing

DNA extraction and PCR amplification of 16S rRNA genes were performed following a procedure described previously (Wang et al. 2020). Briefly, the fecal samples were extracted for DNA using a Qiagen Rneasy Power Microbiome kit (Gryp et al. 2020). Partial 16S rRNA genes were PCR amplified using extracted fecal DNA as templates with 515F and 806R primers (V3-V4 region) (Muyzer et al. 1993). The initial step of PCR started with the denaturation temperature of 95°C for 3 min which was then followed by 30 cycles of 95°C for 60 sec, 57°C for 60 sec, 72°C for 60 sec, and then final extension temperature of 72°C for 10 minutes. To confirm the success of PCR amplification, the size of PCR amplicons was examined using gel electrophoresis. DNA samples with successful PCR amplifications were selected for sequencing, which was outsourced to Novogene (Sacramento, California, USA) using the MiSeq Illumina sequencing platform.

Microbiome Bioinformatic Analyses

The 16S rRNA gene sequencing data were processed by using Qiime 2 (Bolyen et al. 2019; Callahan et al. 2016). Raw reads in FASTQ format were analyzed, filtered and quality controlled based on quality scores using DADA2 in QIIME 2 (Bolyen et al. 2019; Callahan et al. 2016). Taxonomic assignment of amplicon sequence variants (ASVs) was completed using green genes database in QIIME 2 based on paired high-quality sequence reads (Nearing et al. 2018).

Statistical Analysis

Statistical analysis was conducted using QIIME 2 outputs in R Studio version 4.2.1 (Phyloseq Package) (McMurdie & Holmes, 2013; Xia et al. 2023). Alpha-diversity indices including Observed, Chao1, Shannon and Simpson, were calculated based on the ASV level sequence annotations using R package (Vegan) to understand the bacterial diversity of samples during the study period (Oksanen, 2013). For the difference in diversity of microbial communities on different sampling days, a pairwise Wilcoxon test was performed after the Kruskal-Wallis analysis was performed, which is essential for a study with unequal sample sizes (Panek et al. 2018). Principal Coordinate Analysis (PCoA) based on weighted UniFrac distances was performed to identify similarities between the distribution patterns of microbial communities among samples using Vegan and ggplot2 packages in R software (Arafat et al. 2017).

The Analysis of Similarity (ANOSIM) of microbial communities was performed in R using the microbiome package (Lahti & Shetty, 2018) to analyze the similarities between samples from different treatment groups. Taxonomic relative abundance analyses were performed by using top 25 genera and families. To test the significance of the results, One-way ANOVA was performed. Additionally, the Differential Abundance Analysis was performed using DESeq2 package on top 10 most depleted genera overall throughout the study and then in combination of each treatment and sampling day to understand if there were microbial shifts in the gut microbiome communities associated with treatment and timeline of exposure (Love et al. 2014).

RESULTS

The high throughput sequencing generated a total of 7,803,124 sequence reads of the V3-V4 region of the 16S rRNA gene from the total of 72 samples, each library contained 101,340 to 183,053 sequences (102,672.7 in average, with 395-450bp of average sequence length). These reads were processed using the DADA2 QIIME 2 pipeline, and none were excluded as they were all high-quality sequences. After the quality control, the trimmed sequences (≥Q20) were joined, and we obtained 67,469 to 145,379 high-quality reads for analysis for each sample library (Table 1).

Over 99% of total reads were annotated at the phylum level, the percentages were 78% at the family level, 62.4% at the genus level and 48.42% at the ASV level, and the average % coverage was found to be at 97.92±0.4 at any of the level for each sample (from Table 2).

Table 1. Distribution of the number of reads and number of unique ASVs annotated at different taxonomic levels.

Treatment	Day	Input reads	High Quality reads	Phylum ASVs	Family ASVs	Genus ASVs
0μg/kg	0	137744.5± 27570.5	103001.4± 30990.7	436.2± 9.8	357± 4.2	451.5± 2.1
500 μg/kg	0	111167.5± 18227.3	72764.2± 11642.5	322± 63.2	332± 54.3	325.3± 35.4
50 μg/kg	0	134668.3± 25249.4	92187.8± 22562.2	350.8± 26.6	419± 28.2	360.8± 20.2
0μg/kg	7	130941.7± 19707.5	93983.5± 18083.1	403.3± 117.5	296.6± 78.4	417.5± 37.0
500 μg/kg	7	135424± 32052.6	105585.4± 29800.7	325.4± 63.2	380± 54.3	329.9± 35.4
50 μg/kg	7	129050.8± 25284.7	90945.6± 24702.9	362± 45.3	355± 50.2	368.5± 33.4
0μg/kg	14	134589.1± 31738.2	101424.1± 32609.7	418.1± 104.5	374± 37.3	431.1± 75.9
500 μg/kg	14	126473.1± 34928.2	90348.1± 35791.5	282.1± 59.0	349± 44.1	285.1± 22.4
50 μg/kg	14	120572± 19253.2	90945.6± 17146.4	362± 38.7	355± 27.7	368.5± 13.3
0μg/kg	21	131387.6± 45682.7	96819.7± 39451.3	355.6± 171.7	387.1± 120.3	362.6± 59.0
500 μg/kg	21	89395.3± 8922.3	87257.1± 6203.2	353.8± 68.6	362± 56.9	363.2± 29.2
50 μg/kg	21	118127.5± 13689.5	83816.6± 9293.8	355.1± 91.1	363± 67.7	361.3± 32.3

Table 2. Unique ASVs, diversity indices based on ASVs and coverage of the sequencing data from the fecal samples on different sampling days for different treatment groups. The results are presented in the Mean ± Standard deviation format for all the values listed in the table. % Coverage = Observed/Chao1

Sample	**Day**	**ASVs**	**Chao1**	**Shannon**	**Simpson**	**% Coverage**
0µg/kg	0	436.2±115.7	451.5±135.4	3.5±1.1	0.82±0.2	96.9±1.4
500 µg/kg	0	322±56.6	325.3±60.0	3.3±0.2	0.89±0.0	99±0.9
50 µg/kg	0	350.8±39.1	360.8±43.5	4.1±0.2	0.96±0.0	97.8±1.7
0µg/kg	7	403.3±147.4	417.5±162.5	2.9±0.9	0.77±0.2	97.3±2.5
500 µg/kg	7	325.4±40.5	329.9±41.2	3.8±0.4	0.93±0.0	98.4±0.8
50 µg/kg	7	362±150.2	368.5±173.1	3.5±0.4	0.90±0.0	98.3±3.3
0µg/kg	14	418.1±28.9	431.1±29.3	3.7±0.3	0.93±0.0	96.4±1.4
500 µg/kg	14	282.1±34.5	285.1±34.7	3.4±0.2	0.92±0.0	98.9±0.8
50 µg/kg	14	362±85.2	368.5±89.4	3.5±0.6	0.90±0.0	98.3±1.2
0µg/kg	21	355.6±72.9	362.6±74.8	3.8±0.4	0.93±0.0	98.1±0.0
500 µg/kg	21	353.8±64.7	363.2±70.2	3.6±0.3	0.91±0.0	97.5±0.0
50 µg/kg	21	355.1±48.4	361.3±0.2	3.6±0.2	0.91±0.0	98.3±0.0

Taxonomic Composition Analyses

Phylum level analysis

The distribution of the top 25 most abundant phyla (80.65% of sequences assigned at the phylum level) in the gut microbiota across and between different treatment groups were examined (Figure1). Among these abundant phyla, a few showed significant variations among the study groups or over time within a study group.

For *Actinobacteria*, its relative abundance significantly increased during study in all three study groups, i.e., control, 50 μg/kg, and 500 μg/kg treatment groups. However, the 500 μg/kg treatment group had the highest extent of increase, and the relative abundance changed from 0.600% on Day 0 to 6.63% on Day 21 ($P<0.05$). The value was also significantly higher than that in both of the 50 μg/kg treatment group and the control group ($P <0.05$) on Day 21.

For *Bacteroidetes*, the control group exhibited a prevalence of 24.6% on Day 0, with a modest yet significant dip to 18.8% by Day 21 ($P<0.05$). Similar reduction pattern was found for both treatment groups. The 50 μg/kg treatment group started at 28.3% on Day 0 and decreased to 18.7% by Day 21 ($P<0.05$). In the 500 μg/kg treatment group, *Bacteroidetes* were initially found at 32.1% on Day 0, but this reduced to 24.1% by Day 21 ($P<0.05$). The 50 μg/kg treatment had a significant decrease on Day 21 when compared with the control ($P<0.05$), however, 500 μg/kg treatment had similar trend as the control group throughout the study (not significant).

For *Firmicutes*, in the control group, there was a dominance of 67.9% on Day 0, which declined to 23% by Day 21 ($P<0.05$). Similarly, a reduction pattern was found for the 50 μg/kg treatment group (62.3% on Day 0 to 42.2% by Day 21; $P<0.05$) and the 500 μg/kg treatment group (48.9% on Day 0 to 27.9% by Day 21; $P<0.05$). On day 21, the reduction more significant in the

control group($P<0.05$). There was significant difference in abundance in the treatment groups 500 μg/kg and 50 μg/kg on day 21 when compared with the control ($P<0.05$).

Verrucomicrobia displayed minimal changes in the control group during the experiment, however, there were noteworthy changes in both the treatment groups. The abundance in 50 μg/kg group started at a mere 1.86% on Day 0 but surged to 15.1% by Day 14 ($P<0.05$) and underwent fluctuations afterward. In the 500 μg/kg group, *Verrucomicrobia* initiated at 6.03% on Day 0, peaked impressively to 52.3% on Day 14 ($P<0.05$), but then experienced a precipitous drop to 7.1% by Day 21.

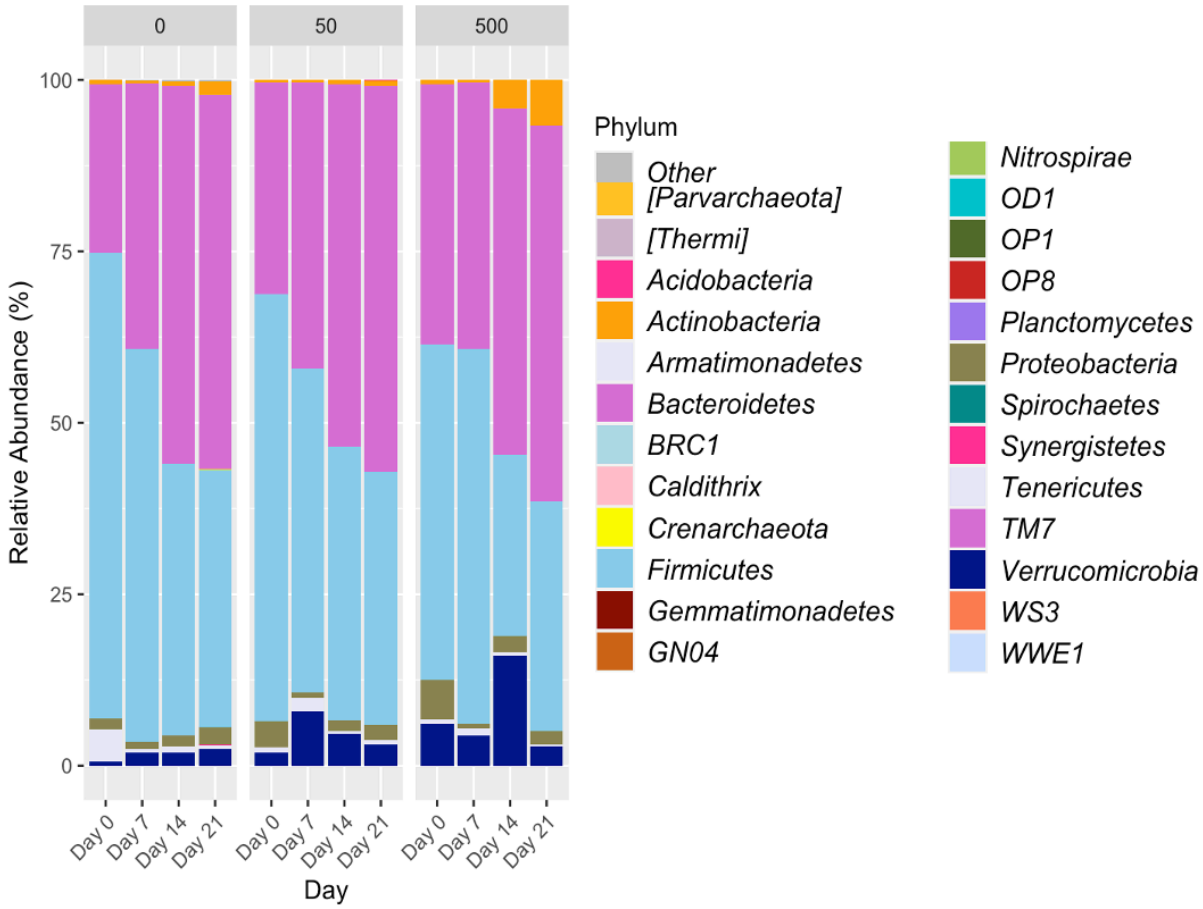

Figure 1. The % relative abundance of the top 25 phyla and other phyla in the samples on different days of sample collection. The X-axis represents the sampling days, and Y-axis represents the % relative abundance, and the facets represent the treatment groups.

Family level analysis

Among abundant families, a few showed significant variations among the study groups or over time within a study group.

For *Bifidobacteriaceae*, a member of the *Actinobacteria* phylum, milder but still significant change pattern was found for both treatment groups when compared with the control. The control group exhibited a 0.02% on Day 0, with a modest yet significant change to 0.6% by Day 21 ($P<0.05$). The 50 µg/kg treatment group started at 0% on Day 0 and increased to 0.04% by Day 21 ($P<0.05$). In the 500 µg/kg treatment group, *Bifidobacteriaceae* were initially found at 0.12% on Day 0, but this decreased sharply to 0.6% by Day 21 ($P<0.05$).

For *Clostridiaceae*, a member of the *Firmicutes* phylum, the control group exhibited a 0.15% on Day 0, with a mild increase to 0.17% by Day 21 ($P<0.05$). An increase pattern was found for 50 µg/kg treatment group too, it was started at 0.02% on Day 0 and increased to 0.33% by Day 21 ($P<0.05$), and this increase was significantly more than that in the control ($P<0.05$). In the 500 µg/kg, the change in *Clostridiaceae* was found to be consistent with the control group. They were initially found at 0.19% on day 0 which was followed by significant but mild increase over the study period and found to be 0.21% on the final day ($P<0.05$).

For *Verrucomicrobiaceae*, a member of the *Verrucomicrobia* phylum, its relative abundance significantly decreased during the study in the control group. The abundance significantly decreased in the treatment groups i.e., 50 µg/kg, and 500 µg/kg. The control group exhibited a 0.6% on Day 0, with a modest yet significant change to 2.4% by Day 21 ($P<0.05$). The 50 µg/kg treatment group started at 1.8% on Day 0 and increased to 3.0% by Day 21 ($P<0.05$). In the 500 µg/kg treatment group, *Verrucomicrobiaceae* were initially found at 6% on Day 0, but this

was drastically decreased to 3% by Day 21 ($P<0.05$). These changes on Day 21 for both treatment groups were found to be significant when compared with the control group ($P<0.05$).

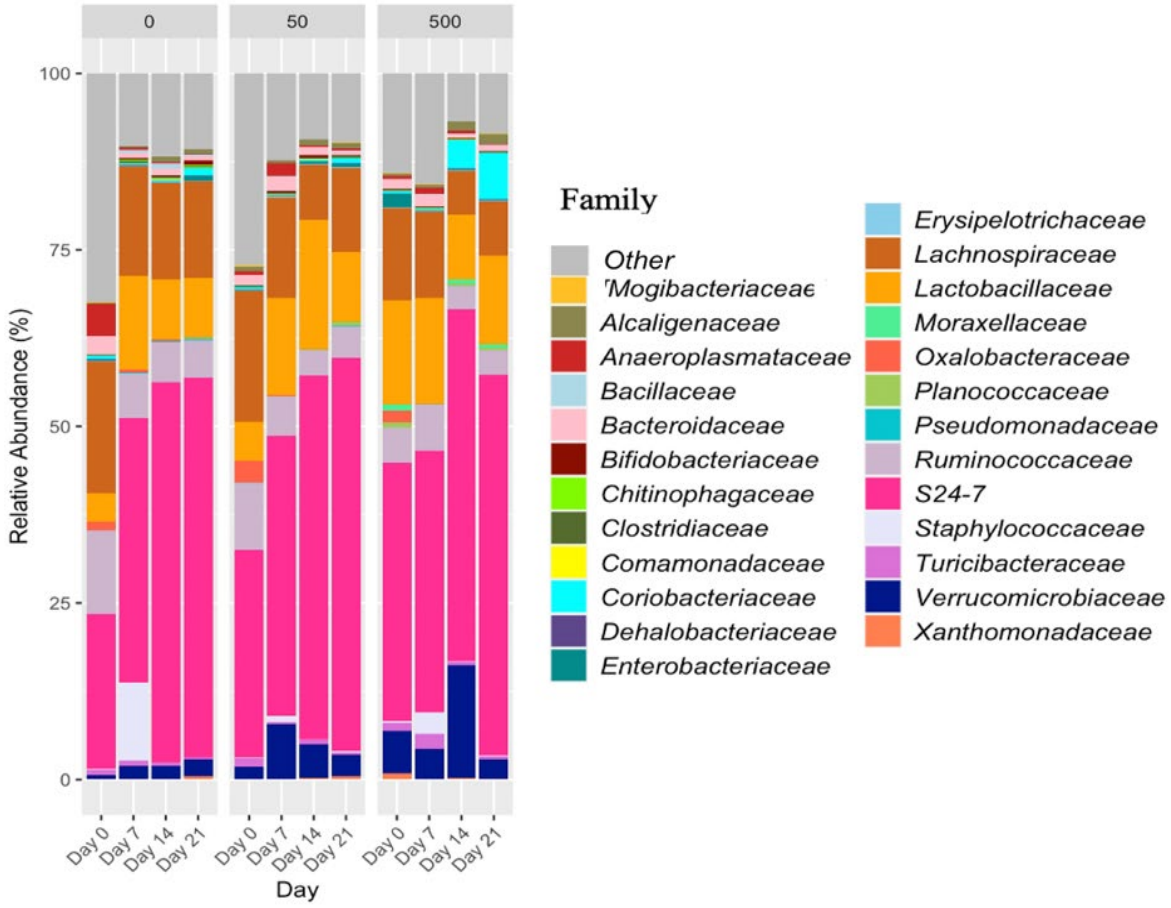

Figure 2. The % relative abundance of the top 25 families and other families in the samples and their proportion in different treatment groups on different days of sample collection. The X-axis represents the sampling days, and Y-axis represents the % relative abundance, and the facets represent the treatment groups.

Genus level analysis

Among abundant genera, a few showed significant variations among the study groups or over time within a study group.

Akkermansia was found to be in increasing in the control group (0.63% on day 0 to 2.4% on day 21, $P<0.05$), but it showcased a significant increase in the treatment group 50 μg/kg (1.8% on day 0 to 3.8% on day 21, $P<0.05$) and the treatment group 500 μg/kg (1.86% on Day 0 to 7.89% on Day 21, $P<0.05$). The change that was more pronounced compared with the control group ($P<0.05$). There was significant increase in abundance in the treatment group 50 μg/kg when compared to the control group ($P<0.05$).

For *Clostridium*, the control group had stable abundance throughout the study (0.04% on day 0 to 2.9% on day 21, $P<0.05$), the treatment group 500 μg/kg saw an increase from 0.06% on day 0 to 0.74% on day 21, ($P<0.05$), which was significant when compared to the control ($P<0.05$). In contrast, the treatment group 50 μg/kg experienced a rise from 0.03% on day 0 to 0.46% on day 21 ($P<0.05$), which was found to be significant when compared with control.

For *Lactobacillus*, the abundance increased in the control group (4% on day 0 to 9% on day 21, $P<0.05$) but decreased in both treatment groups. For the treatment group 500 μg/kg (18.3% on day 0 to 9.96% on day 21, $P<0.05$), the decrease was significant when compared to the control group ($P<0.05$) and the treatment group 50 μg/kg exhibited a decrease in *Lactobacillus* from 11.2% on day 0 to 8.34% on day 21 ($P<0.05$), which was found to be significant when compared to the control. Other changes found were insignificant.

For *Oscillospira*, initial abundances were similar across the control group, 50 μg/kg, and 500 μg/kg treatment groups, which was 7.2%, 5.9% and 2.6% respectively. The control group had declining pattern by the end of the study (from 7.2% on day 0 to 1.9% on day 21, ($P<0.05$)),

however, both of the treatment groups 50 μg/kg (from 5.9% on day 0 to 2.2% on day 21($P<0.05$)), and 500 μg/kg (from 2.6% on day 0 to 1.7% on day 21($P<0.05$)) had less decline throughout the study when compared with the control ($P<0.05$). The changes in both the treatment groups were found to be significant when compared with the control ($P<0.05$).

For *Ruminococcus*, the control, 50 μg/kg, and 500 μg/kg treatment group showed similar abundance on Day 0, which was found to be 2.4%, 1.5%, and 1.1% respectively. The control group had declining pattern by the end of the study (from 2.4% on day 0 to 0.5% on day 21, ($P<0.05$)), however, both of the treatment groups 50 μg/kg (from 1.1% on day 0 to 0.6% on day 21), and 500 μg/kg (from 1.1% on day 0 to 0.6% on day 21, ($P<0.05$)) had less decline compared the control. These changes for both of the treatment groups were found to be significant when compared with the control ($P<0.05$).

Differential abundance analysis showed that *Enterococcus* was found to be significantly depleted in the treatment group 500 μg/kg when compared with 50 μg/kg for the changes between day 0 and 14 (Table 4). However, *Lactobacillus* was found to be significantly depleted only on day 14 for the treatment group 500 μg/kg compared with treatment group 50 μg/kg ($P<0.05$).

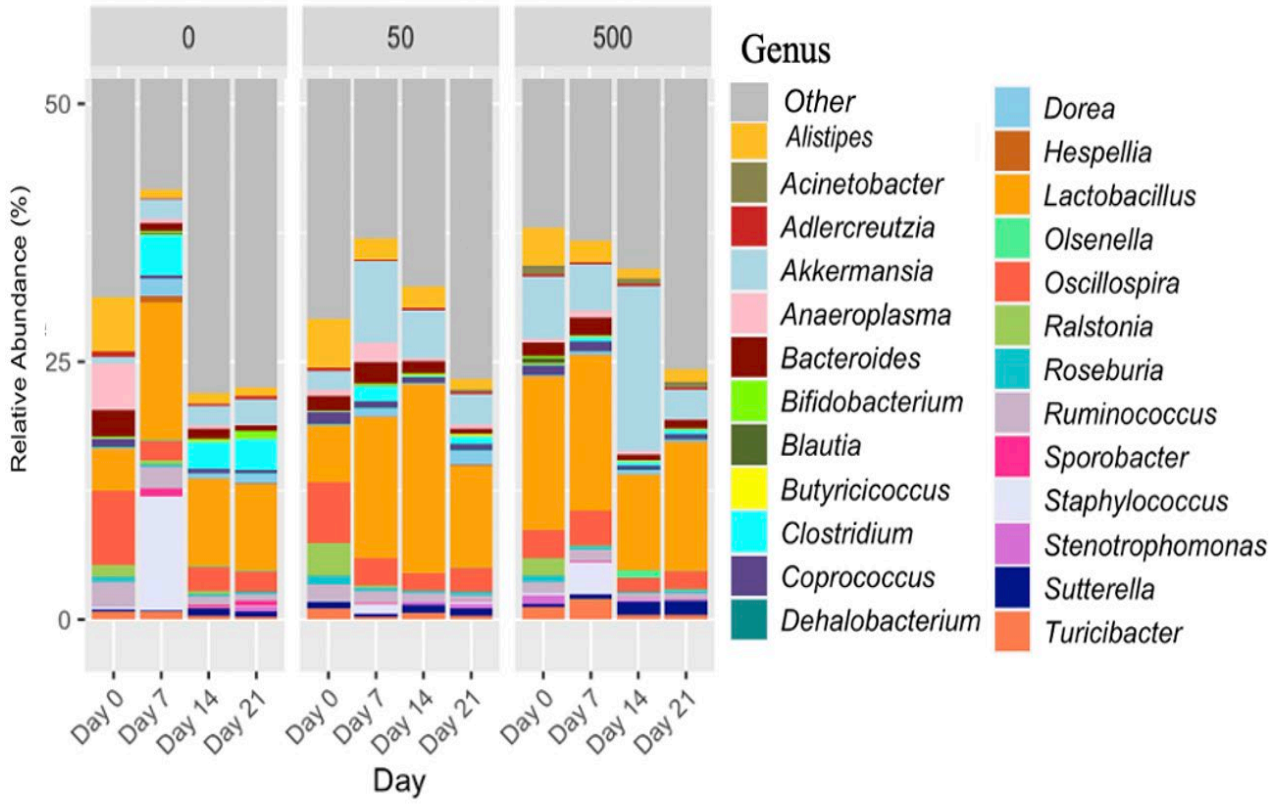

Figure 3. The % relative abundance of the top 25 genera and other genera in the samples and their proportion in different treatment groups on different days of sample collection. The X-axis represents the sampling days, and Y-axis represents the % relative abundance, and the facets represent the treatment groups.

Table 3. Significant results in overall alterations in the Differential Abundance Analysis shows depletion and increase in genera over time. The interpretation of depletion is based on the p-adjusted values.

Treatment Groups	**Days**	**Genus**	**P-Values**	**P-adj**	**Significant change**
50 µg/kg and 500 µg/kg	0 vs 21	*Dorea*	P< 0.05	< 0.05	Increase
50 µg/kg and 500 µg/kg	0 vs 21	*Deinococcus*	P< 0.05	< 0.05	Decrease
50 µg/kg and 500 µg/kg	0 vs 21	*Anaerobaculum*	P< 0.05	< 0.05	Decrease
50 µg/kg and 500 µg/kg	0 vs 21	*Azospirillum*	P< 0.05	< 0.05	Decrease
50 µg/kg and 500 µg/kg	0 vs 21	*Skermanella*	P< 0.05	< 0.05	Decrease
50 µg/kg and Control	0 vs 21	*Vagococcus*	P< 0.05	< 0.05	Increase
50 µg/kg and Control	0 vs 21	*Bifidobacterium*	P< 0.05	< 0.05	Increase
50 µg/kg and Control	0 vs 21	*Akkermensia*	P< 0.05	< 0.05	Decrease

Table 4. Significant results in the Differential Abundance Analysis showing top depleted genera over time in different treatment groups. The interpretation of depletion is based on the p-adjusted values. A p value lower than 0.05 is considered a significant change in abundance.

Treatments Compared	**Days Compared**	**Genus**	**P-Values**	**P-adjusted**	**Depletion**
500 µg/kg vs 50 µg/kg	0 vs 14	*Enterococcus*	P< 0.05	< 0.05	Significant
500 µg/kg vs 50 µg/kg	0 vs 14	*Lactobacillus*	P< 0.05	< 0.05	Significant
500 µg/kg vs 50 µg/kg	7 vs 14	*Enterococcus*	P< 0.05	< 0.05	Significant
500 µg/kg vs 50 µg/kg	14 vs 21	*Enterococcus*	P< 0.05	< 0.05	Significant
500 µg/kg vs 50 µg/kg	0 vs 21	*Enterococcus*	P< 0.05	< 0.05	Significant
500 µg/kg vs 50 µg/kg	7 vs 21	*Enterococcus*	P< 0.05	< 0.05	Significant
500 µg/kg vs 50 µg/kg	14 vs 21	*Enterococcus*	P< 0.05	< 0.05	Significant

Table 5. Significant Differential Abundance Analysis showing top increased taxa over time in different treatment groups. The interpretation of depletion is based on the p-adjusted values. A p value lower than 0.05 is considered a significant change in abundance.

Treatments	**Days**	**Genus**	**P-adjusted**	**P-Values**	**Increase**
500 µg/kg vs 50 µg/kg	14 vs 21	*Akkermansia*	< 0.05	P< 0.05	Significant
500 µg/kg vs 50 µg/kg	0 vs 21	*Akkermansia*	< 0.05	P< 0.05	Significant
500 µg/kg vs 50 µg/kg	7 vs 21	*Akkermansia*	< 0.05	P< 0.05	Significant
500 µg/kg vs 50 µg/kg	14 vs 21	*Akkermansia*	< 0.05	P< 0.05	Significant
500 µg/kg vs 50 µg/kg	14 vs 21	*Acinetobacter*	< 0.05	P< 0.05	Significant
500 µg/kg vs 50 µg/kg	0 vs 21	*Acinetobacter*	< 0.05	P< 0.05	Significant
500 µg/kg vs 50 µg/kg	7 vs 21	*Acinetobacter*	< 0.05	P< 0.05	Significant
500 µg/kg vs 50 µg/kg	14 vs 21	*Acinetobacter*	< 0.05	P< 0.05	Significant
500 µg/kg vs Control	7 vs 21	*Coprococcus*	< 0.05	P< 0.05	Significant
500 µg/kg vs Control	7 vs 21	*Vagococcus*	< 0.05	P< 0.05	Significant
500 µg/kg vs Control	7 vs 21	*Acinetobacter*	< 0.05	P< 0.05	Significant
500 µg/kg vs Control	0 vs 21	*Coprococcus*	< 0.05	P< 0.05	Significant
500 µg/kg vs Control	0 vs 21	*Vagococcus*	< 0.05	P< 0.05	Significant

Table 6. Average *Bacilli* to *Clostridia* percentage abundance ratio for different treatment groups on different sampling days. The data was rarefied before the ratio was calculated. The average absolute and percentage abundance for all the samples representative of each treatment and day combination was calculated. The significance for the data was tested at $P<0.05$.

Treatment	**Day**	***Bacilli* Percent Abundance**	***Clostridia* Percent Abundance**	**Ratio**
0 µg/kg	Day 0	26.5	86.5	0.30
0 µg/kg	Day 7	52.0	48.0	0.81
0 µg/kg	Day 14	25.7	74.3	0.32
0 µg/kg	Day 21	26.6	73.4	0.32
50 µg/kg	Day 0	17.6	82.4	0.12
50 µg/kg	Day 7	36.0	64.0	0.48
50 µg/kg	Day 14	44.5	55.5	0.91
50 µg/kg	Day 21	29.1	70.9	0.41
500 µg/kg	Day 0	36.7	63.3	0.53
500 µg/kg	Day 7	37.1	62.9	0.59
500 µg/kg	Day 14	35.1	64.9	0.64
500 µg/kg	Day 21	41.0	59.0	0.69

Diversity Analyses

Table 7. Difference in diversity in different treatment groups and on sampling days using Kruskal-Wallis test. The p-values for all diversity indices were above the significance threshold ($P<0.05$).

Alpha Diversity	**Treatment p-value**	**Sampling Days p-value**
Simpson	0.3921	0.3558
Shannon	0.3572	0.2035
Observed species	0.1329	0.3278
Chao1	0.06707	0.3264

There were no significant differences in alpha diversity indices at ASV level, including Simpson, Shannon, observed species abundance, and Chao1, across the sampling days between the treatment groups (Table 7).

As the study progressed, changes in diversity indices varied among the treatment groups and sampling days.

There was a significant decrease of the Chao1 index value bewteen Day 0 and Day 7 samples of the control group, Day 7 and Day 14 samples of 50 μg/kg treatment group, Day 7 and Day 14 of 500 μg/kg treatment group, based on the pairwise Wilcoxon test ($P<0.05$) (Figure 4). The same differences were found for the the Observed ASVs ($P<0.05$; Figure 5).

There was a similar significant decrease in the Simpson indices between the Day 0 and Day 7 sample for the control group and the Day 0 and Day 7 samples for the treatment 50 μg/kg group, Day 7 and Day 14 samples from treatment 500 μg/kg based on the pairwise Wilcoxon test ($P < 0.05$; Figure 6).

Only one significant decrease was found for the Shannon index values of the samples, i.e., treatment group 500 μg/kg between day 7 and day 14 based on the pairwise Wilcoxon test ($P < 0.05$; Figure 7).

When performing beta diversity analysis, at the ASV level, the ANOSIM results showed no significant differences in the microbial population between the groups on most sampling days, except for day 14 where a significant difference was observed ($R = 0.2202$, $p < 0.05$). However, the R value for the day 14 comparison was relatively low (Table 9). At the genus level no significant difference was observed in the microbial communities between the treatment groups or on different sampling days based on ASVs (R range: -0.041 to 0.132; Table 10).

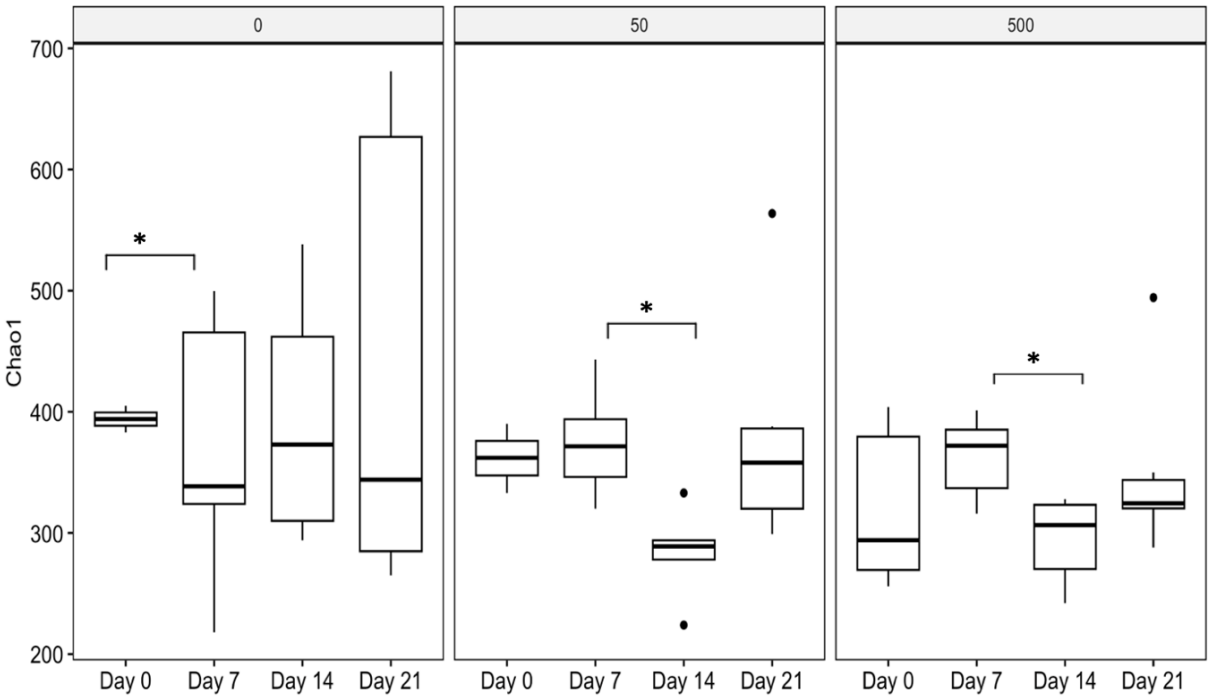

Figure 4. The Alpha diversity analyses on ASV level using Chao1 indices of the gut microbiome across our three treatment study groups on different sampling days.

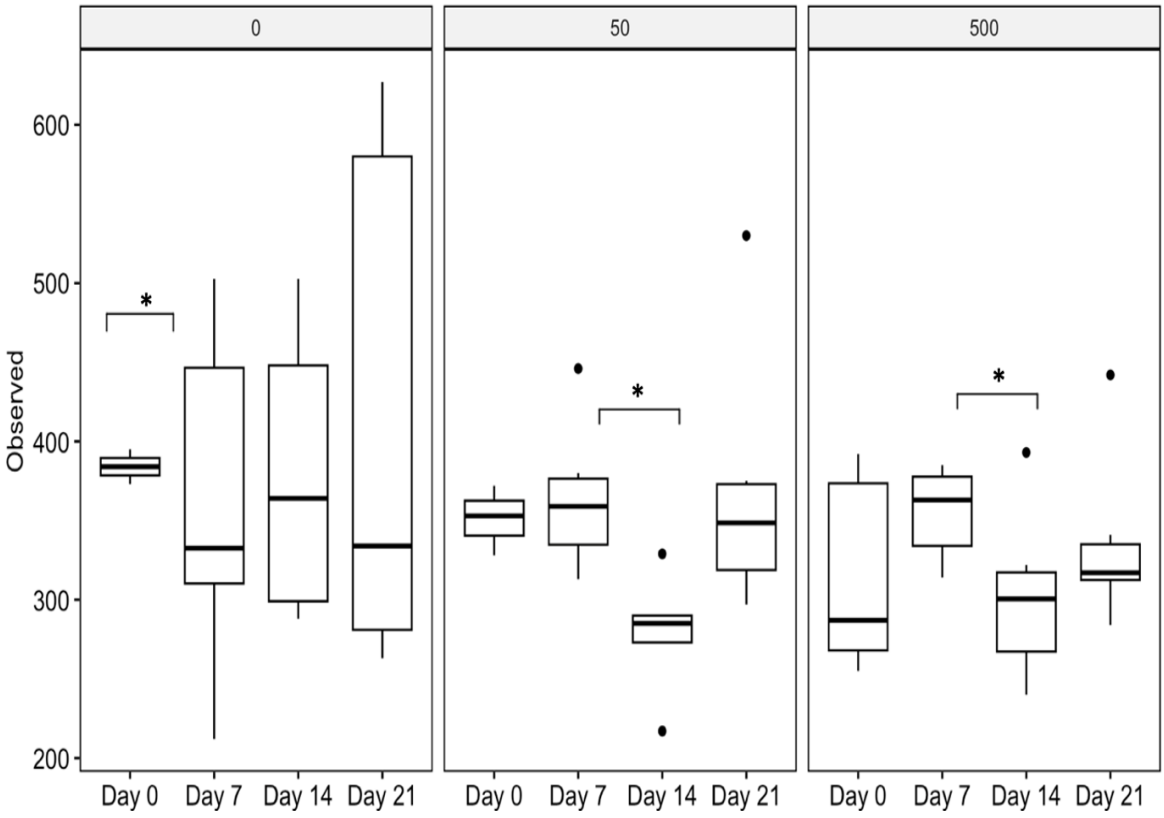

Figure 5. The Alpha diversity analyses on ASV level plot using Observed indices in samples from different treatment groups on different sampling days.

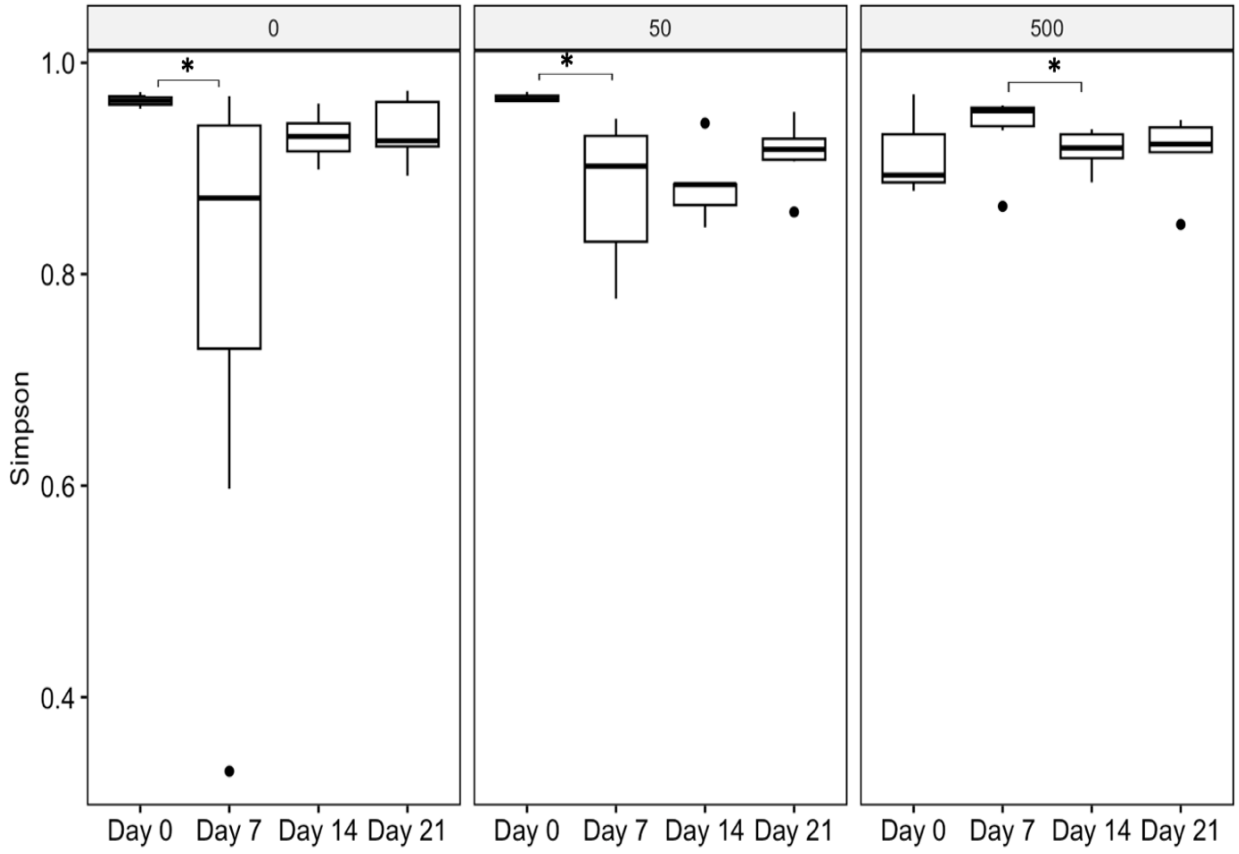

Figure 6. The Alpha diversity analyses on ASV level using Simpson indices in samples from different treatment groups on different sampling days.

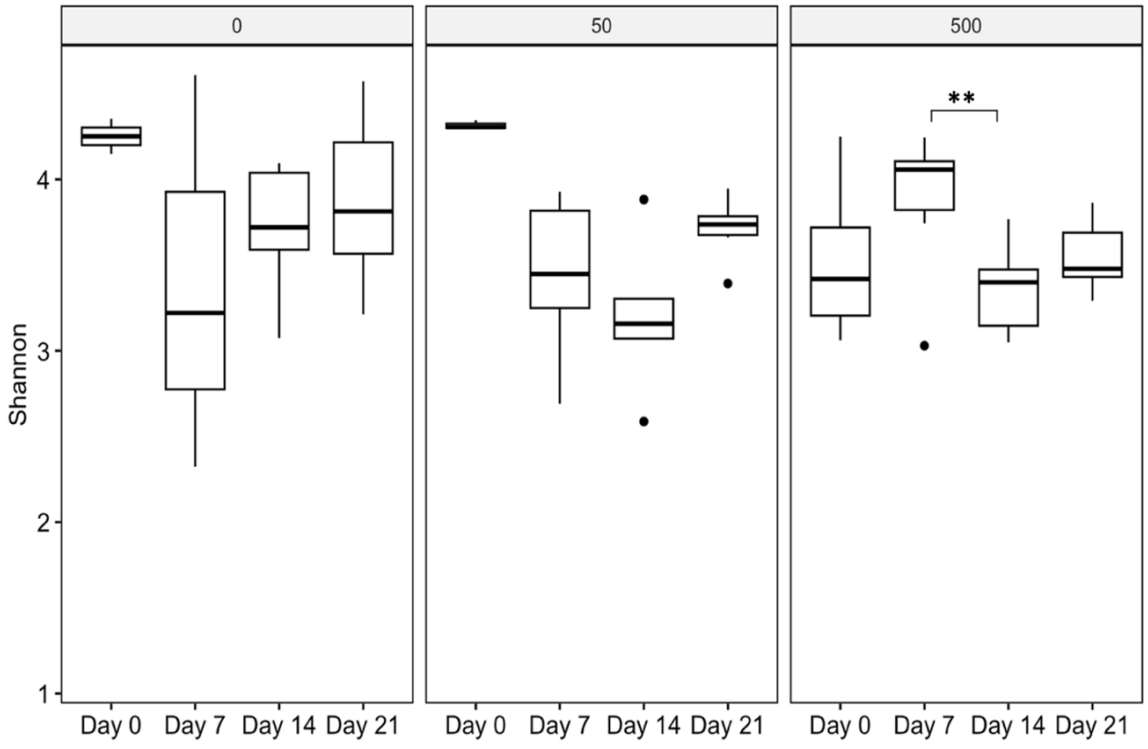

Figure 7. The Alpha diversity analyses on ASV level using Shannon indices in samples from different treatment groups on different sampling days. The asterisks indicate that the differences observed between two points were statistically significant.

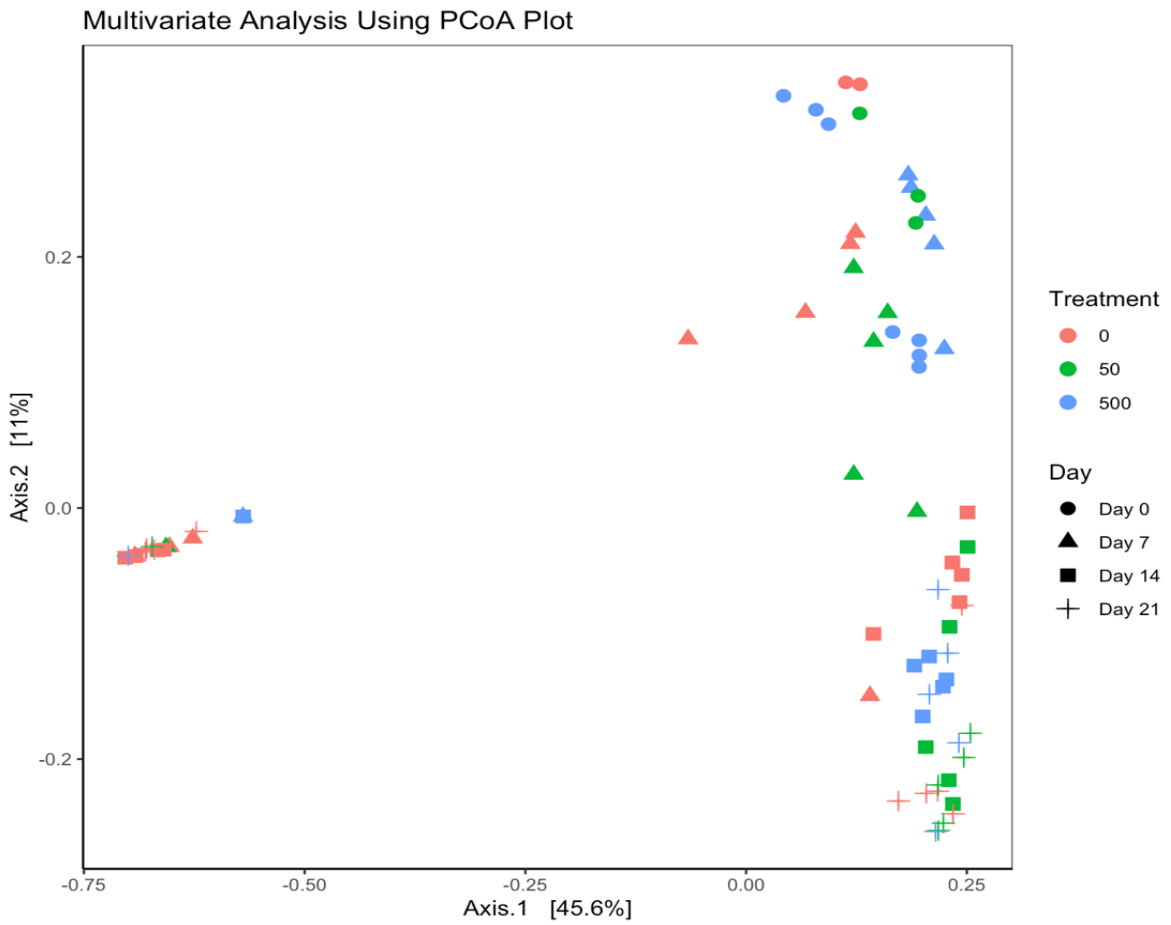

Figure 8. The PCoA (Principal Coordinates Analysis) displays the relationships among microbial communities (at ASV level) from different treatments on different sampling days. The axes represent the principal coordinates that account for the most variation in the data. Axis 1 explains 45.6% of the total variation, while Axis 2 explains 11%.

Table 8. Analysis of Similarities of the microbial populations (based on ASVs) between different treatments on different sampling days.

Treatment/Days	**R Statistic**	**P Value**	**Results**
0µg/kg (Day 0, 7, 14, 21)	0.04421	0.03	No difference
50 µg/kg (Day 0, 7, 14, 21)	0.2906	0.05	Significant difference
500 µg/kg (Day 0, 7, 14, 21)	0.2639	0.03	Significant difference

Table 9. Analysis of Similarities of the microbial populations (based on ASVs) for overall changes on different sampling days.

Sampling Day	**R Statistic**	**P-Value**	**Results**
Day 0 (50 µg/kg, 500 µg/kg, Control)	-0.09463	0.03	No significant difference
Day 7 (50 µg/kg, 500 µg/kg, Control)	0.04075	0.01	No significant difference
Day 14 (50 µg/kg, 500 µg/kg, Control)	0.2202	0.03	Significant difference
Day 21 (50 µg/kg, 500 µg/kg, Control)	0.05114	0.02	No significant difference

Table 10. Similarities of the microbial population (based on genera) between different groups on different days of sampling.

Day/Treatment	**R Statistic**	**P Value**	**Results**
Day 0	0.133	0.04	No difference
Day 7	-0.003	0.01	No difference
Day 14	0.203	0.02	No difference
Day 21	-0.012	0.02	No difference
Treatment 0µg/kg	0.036	0.03	No difference
Treatment 50 µg/kg	0.194	0.01	No difference
Treatment 500 µg/kg	0.299	0.04	No difference

DISCUSSION

The primary objective of this study was to test the hypothesis that short-term exposure to sublethal dosages of MC-LR would trigger gut dysbiosis, substantial alterations in the abundance and diversity of the gut microbiome. The result of this study partially supports the hypothesis that short-term exposure to MC-LR triggers gut dysbiosis. There were a few taxa showed significant changes between treatment and control samples, such as *Firmicutes* and *Bacteroidetes*, two of the dominant phyla present in all groups at the onset of the study. Their abundance significantly decreased in both treatment groups over time compared to the controls (Figure 1). The abundance of *Firmicutes* and *Bacteroidetes* decreased more significantly in groups that received higher doses of MC-LR, suggesting a dose-dependent inverse relationship between treatment and the abundance of these phyla (Turnbaugh et al. 2006).

Actinobacteria showed an increase (Figure 1) in both treatment groups, particularly in the group administered with a higher dose (500 μg/kg), compared with the control. This finding was consistent with a study by Saha et al. (2022), which observed increased relative abundance of *Actinobacteria* in the MC-LR treated (7 μg/ kg body weight) group compared with the control group. This suggests that *Actinobacteria* have a survival advantage or potentially thrive under the altered gut conditions imposed by the MC-LR treatment (Zhang et al. 2015).

Among the genera observed (Figure 3), *Oscillospira and Ruminococcus* showed a sharp decline in the treatment groups compared with the controls. *Oscillospira* has been linked to leanness in humans and has a role in degrading complex carbohydrates (Tims et al. 2018), while *Ruminococcus* is known for its cellulose degradation capability (Flint et al. 2008). The decline of these two genera could indicate a potential disruption in the metabolic function of the gut, leading to subsequent health implications such as obesity.

Lactobacillus had higher relative abundance in both treatment groups than in the control group. This suggests a protective response by this taxon to the disruption caused by the short-term exposure, given that *Lactobacillus* species are known for their beneficial probiotic effects (Bernbom et al. 2012). However, the decline of *Lactobacillus* at Day 21 in the 50 μg/kg group indicates that the gut might not be able to sustain this protective response over time, necessitating further exploration.

The *Bacilli/Clostridia* ratio is a known indicator of the inflammatory response to stress. The control group showed an increase in the *Bacilli/Clostridia* ratio from day 0 to day 7. However, the extent of increase in the *Bacilli/Clostridia* ratio was the highest in the group exposed to the 50 μg/kg from day 0 to day 7 when compared with the control (Table. 6). There was a stable pattern in the in the *Bacilli/Clostridia* ratio in the 500 μg/kg group when compared to the control. This suggests that this toxin dose instigates resilience against the inflammatory response, further indicating the dose-dependent influence on the microbiome composition (Gilbert et al. 2018).

The group administered the higher dose (500 μg/kg) showed more drastic changes in the relative abundance of certain taxa than the other treatment group and controls, such as *Akkermansia*. The species *Akkermansia muciniphila* is known for its beneficial effects on metabolic disorders and its role in maintaining gut barrier integrity (Cani et al. 2013). In a study, researchers discovered that a decline in the protective gut mucus barrier was strongly associated with an increased abundance of *A. muciniphila*. This increase in turn, was associated with the susceptibility to colonization by pathogenic bacteria (Desai et al. 2016). This rise followed by the subsequent fall indicates that short-term exposure may initially trigger an increase in *Akkermansia* in response to gut barrier disruption, but this effect cannot be restored.

Additionally, an increase in the pathogenic genus *Clostridium* in the 500 µg/kg group relative to 50 µg/kg (Figure 3) could indicate a potential elevated risk for infections or gut inflammation at high concentrations (Peterson et al. 2008). This finding highlights the need for further research into the implications of gut microbiota changes for health outcomes, particularly in the context of short-term exposure to environmental stimuli. Moreover, the differential abundance analyses showed that there was a significant depletion of *Enterococcus* in the 500 µg/kg group relative to the 50 µg/kg group, along with temporal depletion of *Lactobacillus* on day 14 followed by recovery on day 21 (Table 4). This presents a compelling picture of the dynamic nature of the gut microbiome and its potential resilience against treatments (Walter & Ley, 2011). This resilience could be a protective mechanism employed by the microbiome in response to environmental perturbations, which is a topic worthy of further research.

The findings from the Kruskal Wallis test performed on the difference in alpha diversity between treatment groups revealed that there were no significant differences in alpha diversity indices between any of the treatment groups and control (Table. 7). This was consistent with a previous study but with a shorter timeline of 7 days (Mills et al. 2021). This suggests a robust and resilient microbiome even under treatment stress (Lozupone et al. 2012). However, a noticeable shift in microbial community structure was found within the higher dose treatment group (500 µg/kg) between Day 7 and Day 14 (Figure 7). The causes behind these significant changes are worth exploring further, and may include host-specific factors, or perhaps additional unidentified environmental variables that could be interacting with treatment exposure (Knight et al. 2011).

CONCLUSION

In conclusion, the result of this study partially supports the hypothesis that short-term exposure to MC-LR triggers gut dysbiosis. Dose-dependent relationship was observed based on

changes in certain taxa as the stronger the dose the greater increase/decrease of their relative abundance was observed in the gut microbiome. However, there was no significant difference observed in the gut microbiome diversity under the exposure of MC-LR. Further investigations into the mechanisms underlying these responses are warranted and could provide valuable insights for therapeutic strategies to mitigate the adverse effects of such exposures on the gut microbiome.

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CHAPTER III

THE IMPACT OF LONG-TERM EXPOSURE TO LOW DOSAGE OF MICROCYSTINS ON MICE GUT MICROBIOME

INTRODUCTION

The increasing prevalence of cyanobacterial blooms, a situation marked by excessive cyanobacteria growth in water bodies, poses significant ecological and public health challenges. Microcystins (MCs), which are produced by some cyanobacterial species like *Microcystis aeruginosa*, are a family of hepatotoxins. The harmfulness of *Microcystis aeruginosa* can be influenced by anthropogenic factors such as agricultural runoff, industrial effluents, and climate change (Giannuzzi & Hernando, 2022; Shen et al. 2003). Among over 100 MC variants, microcystin-leucine-arginine (MC-LR) is the most frequently detected and is recognized for its high toxicity (Corbel et al. 2014).

MC-LR, due to its chemical stability and resistance to degradation, exhibits prolonged persistence in the environment (Charmichael, 1994), it can also be bioaccumulated/biomagnified through trophic transfer along the food chain (Xiang et al. 2020). Safety guidelines, such as the no observed effect level (NOEL) of 1.0 μg/L in the United States, exist (OEPA, 2020). However, various freshwater sites have recorded MC-LR concentrations exceeding these thresholds, contributing to substantial public health concerns (Charmichael et al. 2017; World Health Organization, 2011).

Furthermore, the inability of conventional water treatment facilities to eliminate MCs adds to the growing global problem of these toxins infiltrating drinking water (Mohamed et al. 2015). Humans and animals are primarily exposed to MC-LR through the ingestion of contaminated water or food, inhalation during water-related recreational activities, or dermal contact (Svirčev et al. 2017). In these cases, chronic, frequently low-level, exposure to MC-LR is often observed in real-world scenarios and may lead to cumulative health effects over time (Lee et al. 2020).

Environmental toxins like MC-LR can potentially disrupt the gut microbiome, a complex community of microorganisms crucial for maintaining host health and homeostasis (Ley et al. 2006; Wu and Wu, 2012). The effect of MC on the gut microbiome has been a growing interest in research, and previous investigations have studied the impact of chronic MC-LR exposure on the gut microbiome. Chen et al. (2015) studied the effect of chronic exposure of 200 μg/kg of MC-LR for 3 months on caecum and colon microbiome and found that there was an increase in microbial diversity in MC-LR treated gut microbiome samples compared to the controls. Another study by Lee et al. (2020) adopted a different approach and explored the effects of chronic (36 weeks) MC exposure at 10 μg/kg using a two-stage carcinogenesis mouse model. Their results showed a substantial shift in gut microbiome structure compared to the control group, with outcomes closely mirroring those of the positive cancer group. Zhuang et al. (2021) used MC-LR at concentrations of 1.5 μg/kg and 15 μg/kg for 6 months and their results similarly showed an increase in the overall species diversity of the gut microbiome upon prolonged exposure to MC-LR. In addition, they witnessed a shift associated with decreased abundance of key bacterial taxa such as *Actinobacteria and Bacteroidetes*. A study by Zhao et al. (2020) reported that chronic exposure to the 100 μg/kg, 250 μg/kg, and 750 μg/kg (low, medium, and high dose) of MC-LR in mice showed that prolonged

exposure to 750 µg/kg of MC-LR for 12 months could lead to a high risk for Non-alcoholic fatty liver disease, however, there was no significant difference observed for 100 µg/kg, and 250 µg/kg.

Despite many early studies, our understanding of the influence of chronic MC-LR exposure on the composition of specific microbial taxa at different time intervals remains limited. Despite several studies on the subject, their focus on changes in diversity and compositional change after exposure have missed how those changes progress and persist over time (Chen et al. 2015; Zhuang et al. 2021). In this study, we aimed to fill this knowledge gap by elucidating the effects of long-term exposure to low MC-LR concentrations on the composition and diversity of the gut microbiome in a mouse model. We hypothesized that chronic MC-LR exposure induces significant changes in the microbial diversity and composition over time and has dose-dependent effects on specific bacterial taxa integral to gut health (Yang et al. 2022; Zhuang et al. 2021).

We exposed mice to environmental relevant low doses of MC-LR for a total of 125 days. Fecal samples of mice were collected regularly throughout the exposure period, and high-throughput 16S rRNA gene sequencing was utilized to investigate alterations in the gut microbiome's structural dynamics under chronic MC-LR exposure.

METHODS

Animals and Experimental Groups

Six weeks old C578B16 male (total 21) mice were received from Jackson laboratory-East (Bar Harbor, ME, USA). The process of acclimatization and feeding of animals was done according to the procedure in Chapter II.

The animals were divided into 3 different groups according to the dosage (0 µg/kg bw, 10 µg/kg bw, and 50 µg/kg bw). The animals were weighed every 48 hours right before gavaging toxins orally. Group 1 (G0), the control group was fed with sterile-filtered water, Group 2 (G10)

gavaged with 10 μg/kg bw (low concentration), and Group 3 gavaged with 50 μg/kg bw (high concentration). All the animals were fed and normal feed pellets (Prolab RMH 3000). Three mice were accommodated in each cage. The fecal samples were collected from mice on days 0, 42, 84, and 125 days and they were stored at -80°C until further processed. All the animals had free access to food and water. They were fed with normal feed and water. The feeding was carried out for 126 days and the mice were sacrificed on day 125.

DNA Extraction and 16S rRNA Gene Sequencing

DNA extraction and PCR amplification of 16S rRNA genes were performed following a procedure described previously (Wang et al. 2020). Briefly, the fecal samples were extracted for DNA using a Qiagen Rneasy Power Microbiome kit (Gryp et al. 2020). Partial 16S rRNA genes were PCR amplified using extracted fecal DNA as templates with 515F and 806R primers (Muyzer et al. 1993). The initial step of PCR started with the denaturation temperature of 95°C for 3 min which was then followed by 30 cycles of 95°C for 60 sec, 57 C for 60 sec, 72°C for 60 sec, and then final extension temperature of 72°C for 10 minutes). To confirm the success of PCR amplification, the size of PCR amplicons was examined using gel electrophoresis. DNA samples with successful PCR amplifications were selected for sequencing, which was outsourced to Novogene (Sacramento, California). The samples were amplified using 515F (GTGCCAGCMGCCGCGGTAA) and 907R (CCGTCAATTCCTTTGAGTTT) primers targeting the V4 region before the library preparation and MiSeq Illumina sequencing (16S rRNA gene sequencing) which was performed at the Novogene facility (Sacramento, California, USA).

Sequencing Data Processing and Bioinformatic Analyses

The data received were then processed by using Qiime2. Again, DADA2 pipeline was used for further data processing (Callahan et al. 2015). Alpha diversity metrics, including the Observed,

Chao1, Shannon, and Simpson indices, were computed at the Amplicon Sequence Variant (ASV) level using the dedicated R package (Vegan) (Oksanen, 2013).

This enabled the evaluation of diversity both within individual samples and collectively over the entire sampling duration. To comprehend potential shifts in gut microbiome community composition concerning treatment intensities and exposure duration, differential abundance analysis was carried out. This was performed by using the DeSeq2 package in R (Love et al. 2014). For all the other additional analyses the procedure from Chapter II was followed.

Statistical Analysis

Statistical analyses were conducted using the Vegan and ggplot2 R software packages (Arafat et al. 2017). Comparative evaluations of the microbial community compositions across distinct treatment groups and sampling timelines were performed using one-way ANOVA (Xia & Sun 2017). Principal Coordinate Analysis (PCoA) of bacterial community structure were preformed based on the relative abundance of ASVs using weighted UniFrac distances. To assess the alpha diversity across different treatment groups, the Kruskal Wallis test was employed followed by the pairwise Wilcoxon rank-sum test (Panek et al. ,2018).

RESULTS

Sample reads and sequencing details

The paired-end sequencing of the amplicon targeting the V4 region of the 16S rRNA gene generated a total of 47,000-189,945 sequencing reads per sample (Table 12). After paired end joining and quality control steps 41694-168560 sequencing reads with the average length of 102-255 bp existed in each sample sequence library. A total of 8,455 unique Amplicon Sequence Variants (ASVs) were identified. The ASVs were assigned to a total of 52 distinct phyla, 230

unique families, 387 unique genera, and 278 unique species. The average % coverage was found to be 98.6% in average in all the samples throughout the study period.

Table 11. Alpha Diversity indices, and percentage coverage for different treatment groups. When comparing the changes in each treatment group to the other two groups, significant alterations were observed in multiple microbial parameters.

Sample	**Day**	**Chao1**	**Shannon**	**Simpson**	**% Coverage**
0µg/kg	0	633.3±194	3.6±0.3	0.90±0.0	98.8±0.3
10 µg/kg	0	498±241	3.5±0.7	0.91±0.0	99±0.8
50 µg/kg	0	502.8±160.9	3.8± 0.2	0.94±0.0	98.8±1.0
0µg/kg	42	434.2±59	3.8±0.4	0.93±0.0	99.2±0.5
10 µg/kg	42	590.3±231	4.1±0.2	0.96±0.0	98.9±0.9
50 µg/kg	42	633.8±35	4.4±0.2	0.96±0.0	99.3±0.3
0µg/kg	84	439.5±74	3.8±0.4	0.92±0.0	98.6±0.8
10 µg/kg	84	576.2±164	4.1±0.3	0.95±0.0	99.4±0.2
50 µg/kg	84	508.5±19	4.1±0.1	0.95±0.0	98.4±0.8
0µg/kg	125	490.9±17	3.7±0.2	0.92±0.0	99.1±0.4
10 µg/kg	125	569.2±15	4.6±0.3	0.97±0.0	98.8±0.3
50 µg/kg	125	576.9±37	4.7±0.3	0.97±0.0	99.2±0.4

Table 12. Input raw sequence reads, high quality reads and number of unique ASVs assigned to the Phyla, Families, Genera levels for all the treatment groups.

Sample	Day	High Quality reads	ASVs	Phylum ASVs	Family ASVs	Genus ASVs
0µg/kg	0	148545± 7717.9	629.1±191	609± 219.2	426± 144.1	198.8± 85.2
10 µg/kg	0	150555± 2697.2	493.6±239	479.3± 224.1	336.1± 156.6	175.8± 102.6
50 µg/kg	0	152941± 6431.4	497.5±160	489.8± 146.2	354.6± 99.0	177.8± 55.3
0µg/kg	42	110477± 34359	430.8±58	423± 88.9	297.3± 65.7	139± 34.4
10 µg/kg	42	156167± 12697.2	585.1±232	566.6± 206.7	392.3± 121.5	191± 67.8
50 µg/kg	42	152460.1± 7537.8	629.5±32	612.3± 65.8	409.8± 32.1	187.3± 13.6
0µg/kg	84	138347± 18928.5	434.6±73	428± 68.3	314.3± 43.9	145.6± 23.5
10 µg/kg	84	124384± 6553.4	573.5±164	559± 145.7	389.5± 79.9	181± 29.9
50 µg/kg	84	120138.3± 6553.4	500.8±18	496± 12.8	355.8± 18.7	170.5± 10.8
0µg/kg	125	126855± 13064.6	486.8±17.4	480± 57.6	354.5± 34.9	168.6± 16.1
10 µg/kg	125	124988± 3383.6	563.8±35.3	556± 54.9	401.6± 35.1	187.6± 18.3
50 µg/kg	125	117893± 1933.9	573±38	522± 61.6	370.9± 39.3	173.4± 16.0

Taxonomic Classification

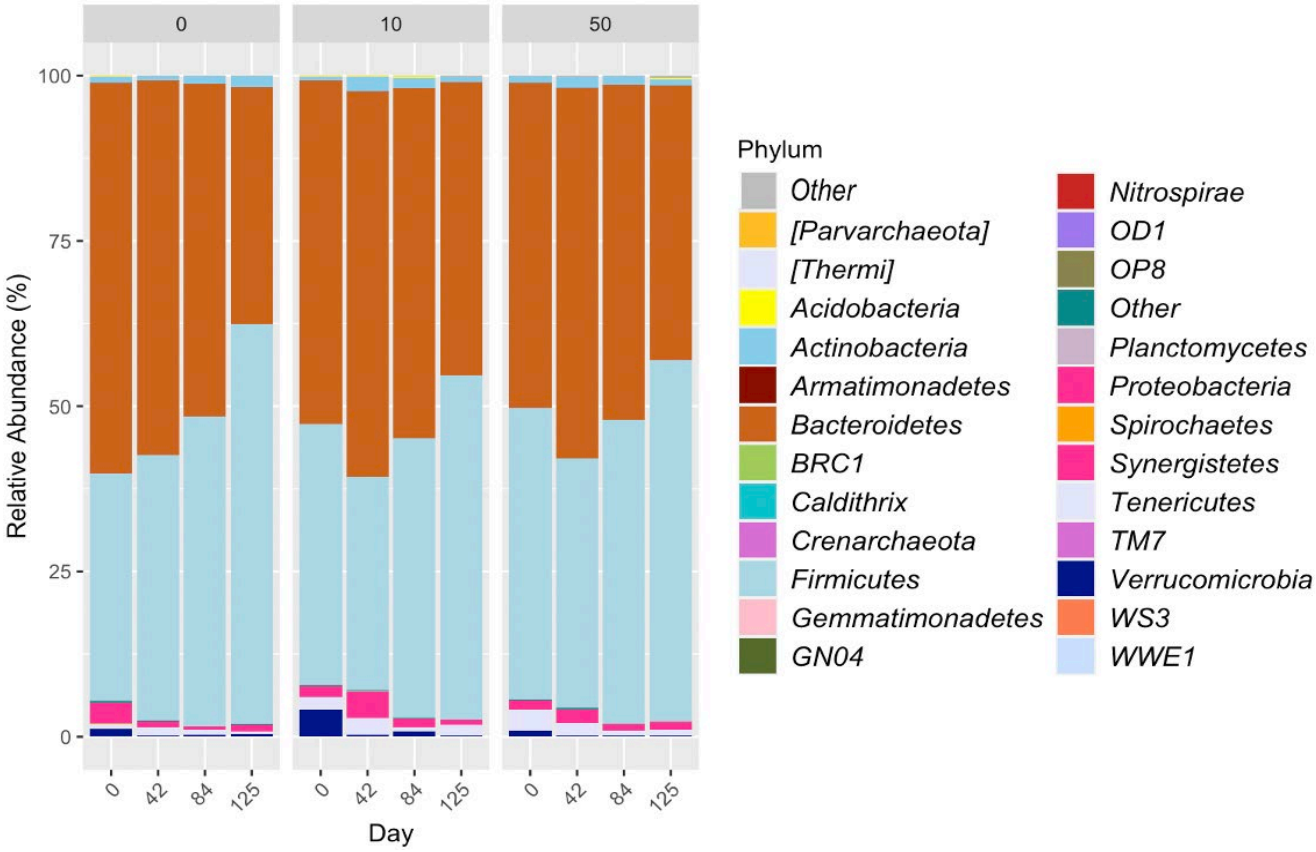

Figure 9. The % relative abundance of the top 25 phyla and other phyla in the samples and their proportion in different treatment groups on different days of sample collection. The X-axis represents the sampling days, and Y-axis represents the % relative abundance, and the facets represent the treatment groups.

Taxonomic distribution at the phylum level

Among abundant phyla, a few showed significant variations among the study groups or over time within a study group.

For *Actinobacteria*, its relative abundance significantly increased during study (from 0.78% on day 0 to 1.72% on day 125, $P<0.05$) in the control, and (0.58% on day 0 to 0.64% on day 125) 10 μg/kg treatment groups. The 50 μg/kg treatment, however, exhibited a significant decrease when compared to itself and the control group, where it changed from 0.98% on Day 0 to 0.78% on Day 125 ($P<0.05$).

For *Bacteroidetes*, its relative abundance significantly decreased during the course of study in all three study groups. The control group exhibited a prevalence of 59% on Day 0, with a modest yet significant reduction to 56% by Day 42, to 50% by Day 84, and 35% on day 125 ($P<0.05$). The 10 μg/kg treatment group started at 51% on Day 0 and decreased to 44% by Day 125 ($P<0.05$). In the 50 μg/kg treatment group, *Bacteroidetes* were initially found at 49% on Day 0, 50% on day 84, but this reduced to 41% by Day 125 ($P<0.05$). The changes in the treatment groups when compared with the control, however, were found to be insignificant.

For *Firmicutes*, its relative abundance significantly increased during the study in the control group (39% on day 0 to 60% on day 125, $P<0.05$), however, the treatment groups experienced significant decrease by day 42, when compared to the control group. The 10 μg/kg treatment group experienced a decrease to 52.0% from 59% on day 42 ($P<0.05$), followed by an increase to 55% on day 125 ($P<0.05$), and the 50 μg/kg group started at 45% on day 0 ($P<0.05$), which settled at 54.5% on day 125 ($P<0.05$). On Day 125, the control group's *Firmicutes* abundance significantly increased to 60.6% ($P<0.05$), becoming the most abundant phyla.

For *Proteobacteria*, the control group had the decreasing pattern throughout the study where it had 3% abundance on Day 0 which decreased to 1% on Day 125 ($P<0.05$). The 10 μg/kg treatment group when compared with the control showed no significant change however, the 50 μg/kg treatment group marked stable pattern when compared with the control group (1.3% on day 0 to 1.8% on day 125) ($P<0.05$) in the abundance of *Proteobacteria*.

For *Tenericutes* the control group showed a mild but significant reduction (0.73% on day 0 to 0.35% on day 125) in the study ($P<0.05$), however, the 10 μg/kg treatment had the least impact (1.8% on day 0 to 1.6% on day 125) maintaining a relatively stable abundance ($P<0.05$). The 50 μg/kg treatment group experienced the most significant decline (3.18% on day 0 to 0.92% on day 125) ($P<0.05$) when compared to the control group.

For *Verrucomicrobia*, the control showed a stable pattern, however, the treatment groups showed a decreasing trend when compared with the control ($P<0.05$). In the control group, the abundance was 1.2% on day 0 which maintained stable pattern to 0.8% by day 125. However, changes were found in the 10 μg/kg treatment (1.2% on day 0 to 0.21% on day 125; $P<0.05$). The 50 μg/kg dosage showed a insignificant changes when compared with the control group throughout the study.

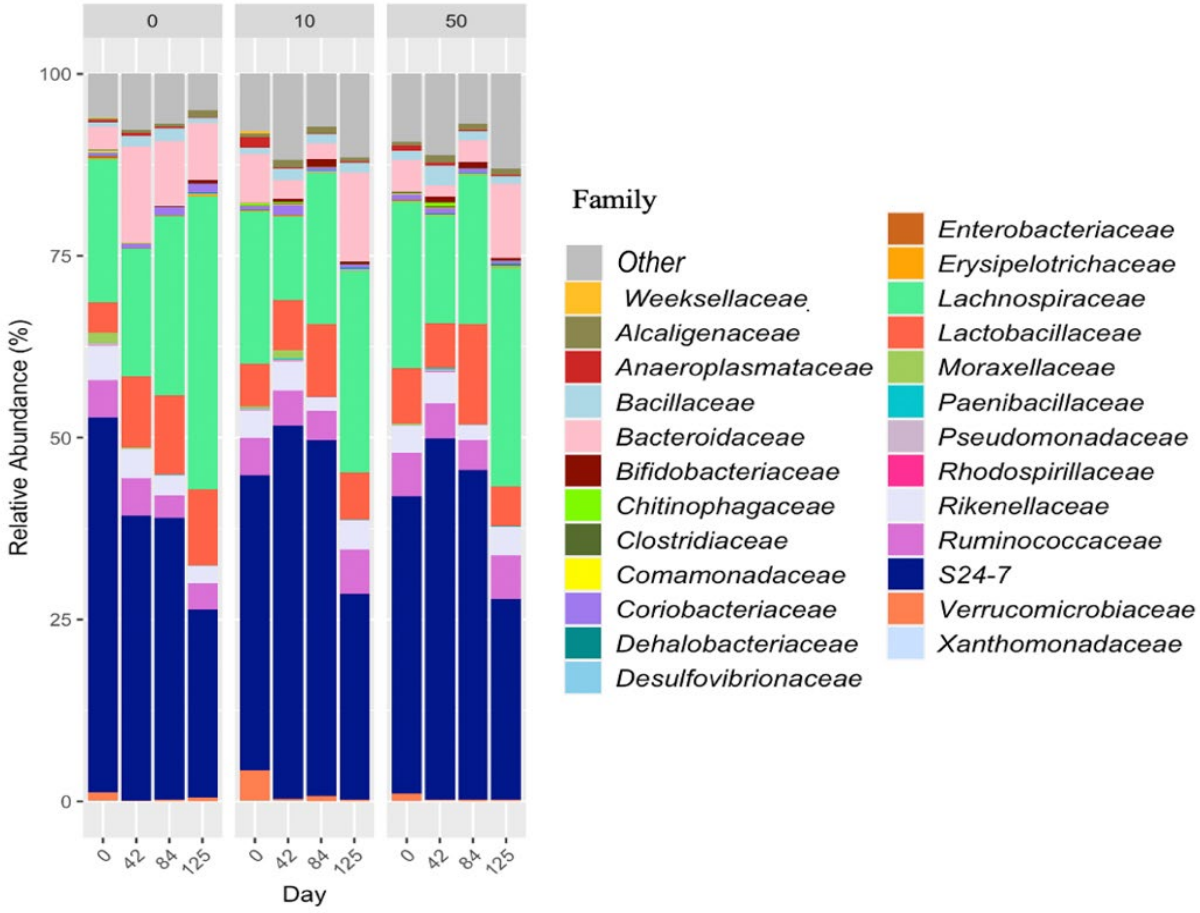

Figure 10. The % relative abundance of the top 25 families and other families in the samples and their proportion in different treatment groups on different days of sample collection. The X-axis represents the sampling days, and Y-axis represents the % relative abundance, and the facets represent the treatment groups.

Taxonomic distribution at the family level

A few of the major families were found to have significant interactions throughout the study period.

The family *Bifidobacteriaceae*, which belongs to the phylum *Actinobacteria,* remained relatively stable in the control group (0.09% on day 0 to 0.08% on day 125), maintaining a consistent abundance from day 0 to day 125. However, in treatment 10 μg/kg, a significant increase (0.03% on day 0 to 0.5% on day 125) was observed in the abundance of *Bifidobacteriaceae* when compared with the control group. The treatment 50 μg/kg showed a significant increase from 0.09% on day 0 to 0.31% on day 125 ($P<0.05$), when compared with the control from day 0 to 125.

Similarly, the family *Clostridiaceae*, which belongs to the phylum *Firmicutes*, showed stability in the control group (0.08% on day 0 to 0.04% on day 125, ($P<0.05$), but in treatment 50 μg/kg, a significant decline was observed (0.15% on day 0 to 0.05% on day 125, $P<0.05$). However, the changes in the 10 μg/kg (0.03% on day 0 to 0.08% on 125) was found to be insignificant when compared to the control group.

For the family *Verrucomicrobiaceae*, which belongs to the phylum *Verrucomicrobia* significant decline in the abundance for both control (1.2% on day 0 to 0.4% on day 125, $P<0.05$) and treatment 10 μg/kg (4% on day 0 to 0.21% on day 125, $P<0.05$). In treatment 50 μg/kg, a similar but significant pattern (1.2% on day 0 and 0.15% on day 125, $P<0.05$) of *Verrucomicrobiaceae* was found when compared with the control group.

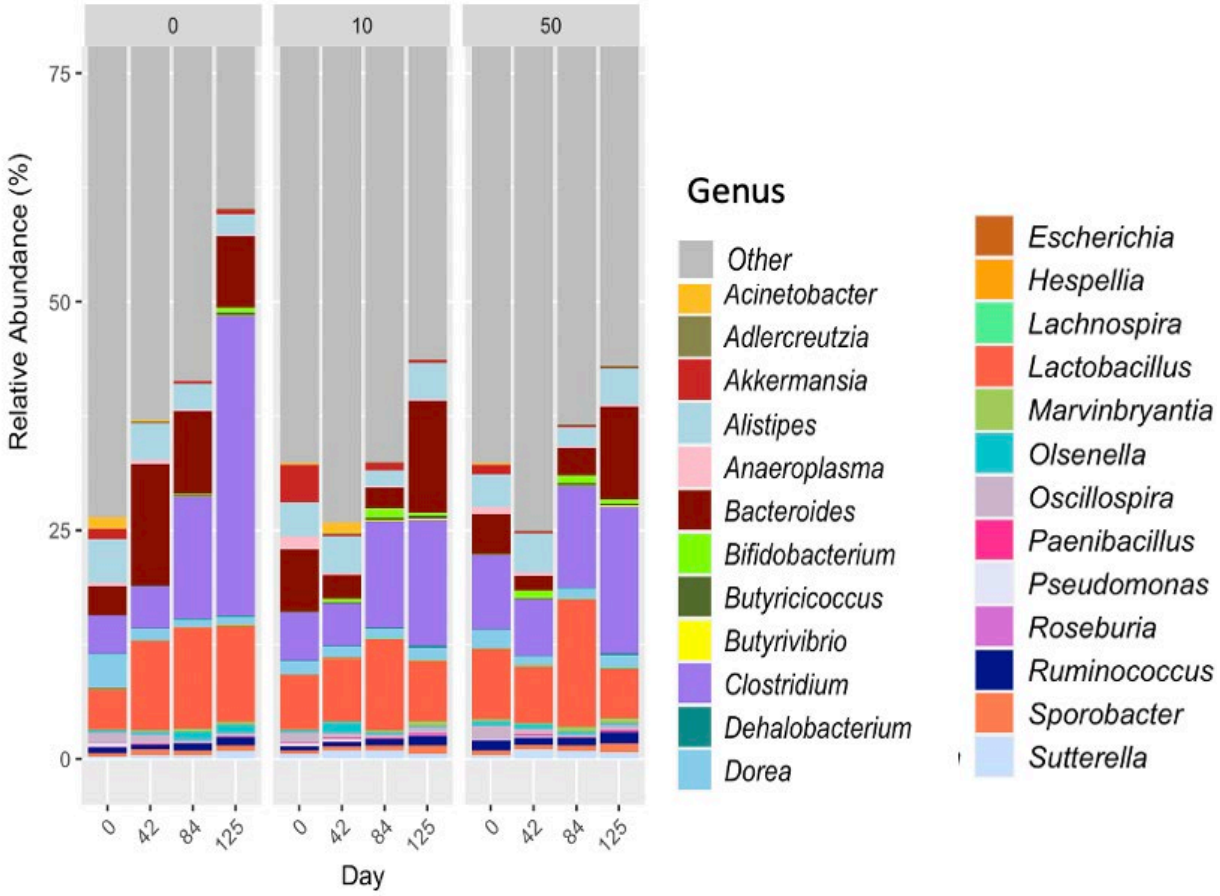

Figure 11. The % relative abundance of the top 25 genera and other genera in the samples and their proportion in different treatment groups on different days of sample collection. The X-axis represents the sampling days, and Y-axis represents the % relative abundance, and the facets represent the treatment groups.

Taxonomic distribution at the genus level

Some genera varied their relative abundance with time and between samples.

Akkermansia was found to maintained decreasing trend throughout the study period in the both the control group (1.2% on day 0 to 0.4% on day 125, $P<0.05$), and the Treatment groups. The treatment 10 μg/kg group (4% on Day 0 to 0.21% on Day 125, $P<0.05$) showed more steeper but significant decline in abundance when compared to Treatment 50 μg/kg group (0.94% on day 0 to 0.15% on day 125, $P<0.05$) and the control group. There was significant difference observed in the Treatment 10 μg/kg group and Treatment 50 μg/kg group when compared to the control ($P<0.05$).

Bacteroides showed an increasing trend in the control group (3.13% on day 0 to 7.6% on day 125, $P<0.05$), and both of the treatment groups. The Treatment 10 μg/kg (6.67% on day 0 to 12.1% on day 125, $P<0.05$) which was found to be significant increase when compared with the control. The treatment 50 μg/kg showed a significant increase from 4.5% on day 0 to 10.2% on day 125 ($P<0.05$), when compared with the control group.

Differential Abundance Analysis

Depletion of beneficial bacteria, namely, *Lactobacillus* and *Bifidobacterium* was identified based on Differential Abundance Analysis (Table 13). Our results showed significant changes (both p-value and p-adjusted<0.05) in the abundance of certain genera over time when comparing different MC-LR exposure levels (Table 14). Specifically, there's a significant depletion of *Butyricicoccus* in the 10 μg/kg vs 50 μg/kg group at both day 42 and 84. When comparing the 50 μg/kg treatment group with the control, *Clostridium* and *Butyricicoccus* displayed significant depletion at days 42 and 84. Additionally, a comparison between the samples

from 50 µg/kg treatment and control on day 125 showed significant changes in the levels of various genera, including *Clostridium, Paenibacillus, Corynebacterium,* and *Sphingobacterium.*

Table 13. Significant results in the Differential Abundance Analysis showing top depleted genera over time in different treatment groups. The interpretation of depletion is based on the p-adjusted values. A p value lower than 0.05 is considered as significant change in abundance.

Treatments Compared	**Days Compared**	**Genus**	**P-Values**	**P-adj**	**Depletion**
0µg/kg vs50 µg/kg	125 vs 0	*Lactobacillus*	P< 0.05	< 0.05	Significant
10 µg/kg vs 50 µg/kg	125 vs 0	*Lactobacillus*	P< 0.05	< 0.05	Significant
0µg/kg vs 50 µg/kg	125 vs 0	*Bifidobacterium*	P< 0.05	< 0.05	Significant
10 µg/kg vs 50 µg/kg	125 vs 0	*Bifidobacterium*	P< 0.05	< 0.05	Significant

Table 14. Significant results in overall alterations in the Differential Abundance Analysis shows depletion in genera over time. The interpretation of depletion is based on the p-adjusted values.

Comparison	**Days Compared**	**Genus**	**p-value**	**p-adj**	**Depletion**
10 µg/kg vs 50 µg/kg	42	*Butyricicoccus*	<0.05	<0.05	Significant
10 µg/kg vs 50 µg/kg	84	*Butyricicoccus*	<0.05	<0.05	Significant
50 µg/kg vs Control	42	*Clostridium*	<0.05	<0.05	Significant
50 µg/kg vs Control	42	*Butyricicoccus*	<0.05	<0.05	Significant
50 µg/kg vs Control	84	*Clostridium*	<0.05	<0.05	Significant
50 µg/kg vs Control	84	*Butyricicoccus*	<0.05	<0.05	Significant
50 µg/kg vs Control	125 vs 125	*Clostridium*	<0.05	<0.05	Significant
50 µg/kg vs Control	125 vs 125	*Paenibacillus*	<0.05	<0.05	Significant
50 µg/kg vs Control	125 vs 125	*Corynebacterium*	<0.05	<0.05	Significant
50 µg/kg vs Control	125 vs 125	*Sphingobacterium*	<0.05	<0.05	Significant

Table 15. Significant results in the Differential Abundance Analysis showing top increased genera over time in different treatment groups. A p-adjusted value lower than 0.05 is significant change in abundance. Statistical significance of these results was further tested at P<0.05.

Comparison	**Days Compared**	**Genus**	**p-value**	**Increase**
50 vs Control	125 Vs 42	*Azospirillum*	<0.05	Significant
50 vs Control	125 Vs 42	*Paenibacillus*	<0.05	Significant
50 vs Control	125 Vs 42	*Pseudomonas*	<0.05	Significant
50 vs Control	125 Vs 42	*Azospirillum*	<0.05	Significant
50 vs Control	125 Vs 42	*Paenibacillus*	<0.05	Significant
50 vs Control	125 Vs 42	*Pseudomonas*	<0.05	Significant
50 vs Control	Day 42	*Azospirillum*	<0.05	Significant
50 vs Control	Day 42	*Paenibacillus*	<0.05	Significant
50 vs Control	Day 42	*Pseudomonas*	<0.05	Significant
50 vs Control	42 vs 125	*Azospirillum*	<0.05	Significant
50 vs Control	42 vs 125	*Paenibacillus*	<0.05	Significant
50 vs Control	42 vs 125	*Pseudomonas*	<0.05	Significant

When comparing the 50 µg/kg treatment group with the control over a period from day 42 to day 125, there was significant increase in the abundance of *Azospirillum, Paenibacillus,* and *Pseudomonas*. The 50 µg/kg treatment group when compared with the control (50 vs 0) at day 42 showed a significant increase in the same three genera, *Azospirillum, Paenibacillus,* and *Pseudomonas* (Table 15).

Table 16. Average ratio of *Bacilli* to *Clostridia* for different treatment groups on different sampling days. The data was rarefied before the ratio was calculated.

Treatment	**Day**	***Bacilli***	***Clostridia***	**Ratio**	**P value**
0	0	7341	41731	0.176	P<0.05
0	42	16111	41432	0.389	P<0.05
0	84	18485	48648	0.380	P<0.05
0	125	16236	70213	0.231	P<0.05
10	0	9739	46822	0.208	P<0.05
10	42	12582	33334	0.377	P<0.05
10	84	16153	44195	0.365	P<0.05
10	125	11403	62920	0.181	P<0.05
50	0	12741	50459	0.253	P<0.05
50	42	13053	40910	0.319	P<0.05
50	84	21706	43960	0.494	P<0.05
50	125	9592	68273	0.140	P<0.05

Comparing the treatment groups with the control group, there were significant changes in the *Bacilli* to *Clostridia*. On day 42, there was a significant increase in the ratio for both treatment groups 10 μg/kg and 50 μg/kg compared to the control group. The 10 μg/kg treatment group showed a percentage increase of 77.48% (P<0.05.) while 50 μg/kg treatment group showed a percentage increase of 27.56% (P<0.05).

The 50 μg/kg treatment group had a significant increase in the ratio of *Bacilli* to *Clostridia* on day 84 when compared to the control group, with a percentage increase of 95.24% (P<0.05). There were no other significant changes observed in the ratio of *Bacilli* to *Clostridia* compared to the control group. On the longitudinal analysis, 10 μg/kg treatment group had decreasing trend in the ratio from day 0 to 125, which decreased by 12.98% (P>0.05), which was not statistically significant. Other comparisons throughout the study yielded insignificant results.

Table 17. Average *Bacteroidetes to Firmicutes* absolute abundance ratio for different treatment groups on different sampling days. The data was rarefied before the ratio was calculated.

Treatment	**Day**	***Bacteroidetes***	***Firmicutes***	**Ratio**
0	0	84744	49161	0.580
0	42	81072	57647	0.711
0	84	71909	67247	0.935
0	125	51198	86768	1.69
10	0	74340	56642	0.762
10	42	83559	46034	0.551
10	84	75879	60576	0.798
10	125	63642	74545	1.17
50	0	70399	63278	0.899
50	42	80207	54093	0.674
50	84	72538	65872	0.908
50	125	59546	78099	1.31

The average absolute and percentage abundance for all the samples representative of each treatment and day combination was calculated. Comparing the *Bacilli* to *Clostridia* ratio at day 0 to day 125 (Table 17.), 50 μg/kg treatment group showed a sharp increase in the ratio when compared to both the control and treatment group 10 μg/kg. The ratio increased from 0.899 to 1.31 ($P< 0.05$). All the values reported were tested at $P< 0.05$ and were found to be statistically significant.

Diversity Analysis

Table 18. Difference in diversity in different treatment groups and on sampling days using Kruskal-Wallis test.

Alpha Diversity Index	**Treatment p-value**	**Sampling Days p-value**
Simpson	0.3921	0.3558
Shannon	0.3572	0.2035
Observed	0.1329	0.3278
Chao1	0.06707	0.3264

Based on the Kruskal-Wallis test, there were no significant differences found in alpha diversity indices between control and the treatment groups on different sampling days (Table 18). The alpha diversity metrics used for this analysis include Simpson, Shannon, observed species abundance, and Chao1.

When performing longitudinal analysis, some differences were observed within the sample study group between the sampling time. The Chao1 indices showed that there was difference between samples from treatment group 50 µg/kg between samples from day 42 and day 84 (Figure 12). The differences were higher between these days compared to the samples from other days. There was a significant difference between the samples from treatment group 50 µg/kg between samples from day 42 and day 84 compared to the difference between samples from day 42 and 125, and day 84 and 125 based on the pairwise Wilcoxon test ($P < 0.05$).

For the Shannon index (Figure. 13), a moderate difference was found in samples from the treatment group receiving 10 µg/kg between day 0 and day 125, and day 42 and day 125. Meanwhile, there was a significant difference in the 50 µg/kg treatment group between samples from day 0 and 42, day 42 and day 84, and day 84 and day 125, as determined by the pairwise Wilcoxon test ($P < 0.05$). There was moderate difference between the samples from treatment

group 10 µg/kg between day 0 and day 125, day 42 and day 125 and significant difference between treatment group 50 µg/kg between samples from day 0 and 42, day 42 and day 84 and day 84 and day 125 based on the pairwise Wilcoxon test ($P < 0.05$).

The Simpson index (Figure 14.) showed a significant difference in the samples from the treatment group receiving 50 µg/kg between day 0 and day 42, day 0 and day 84, and day 84 and day 125. This was confirmed by the pairwise Wilcoxon test ($P < 0.05$). There was significant difference between the samples from treatment group 50 µg/kg between day 0 and day 42, day 0 and day 84 as well as day 84 and day 125 based on the pairwise Wilcoxon test ($P < 0.05$).

For the Observed ASVs (Figure 15), a significant difference was found in the samples from the treatment group receiving 50 µg/kg between day 42 and day 84, again determined by the pairwise Wilcoxon test ($P < 0.05$).

A treatment-induced pattern was visible over time, as distinct community formations were observed based on the treatment dose in the PCoA plot (Figure 16). Treatment 50 µg/kg and Treatment 10 µg/kg were found to have similar microbial community grouped together on day 42, which was drastically different from the control samples (red triangles). On day 125, there were different groups formed for each of the three study groups. Further analyses were done to confirm the results by using Analysis of Similarities (ANOSIM). There was significant difference in the microbial populations between the samples from 10 µg/kg on Day 0 and the samples from 10 µg/kg on Day 125. There was no significant difference in the microbial communities when the comparison was made between days for the treatment groups or when compared with the control group over time ($P <0.05$). From the Table 20, there was no significant difference in the microbial populations between all the treatment groups on any sampling day.

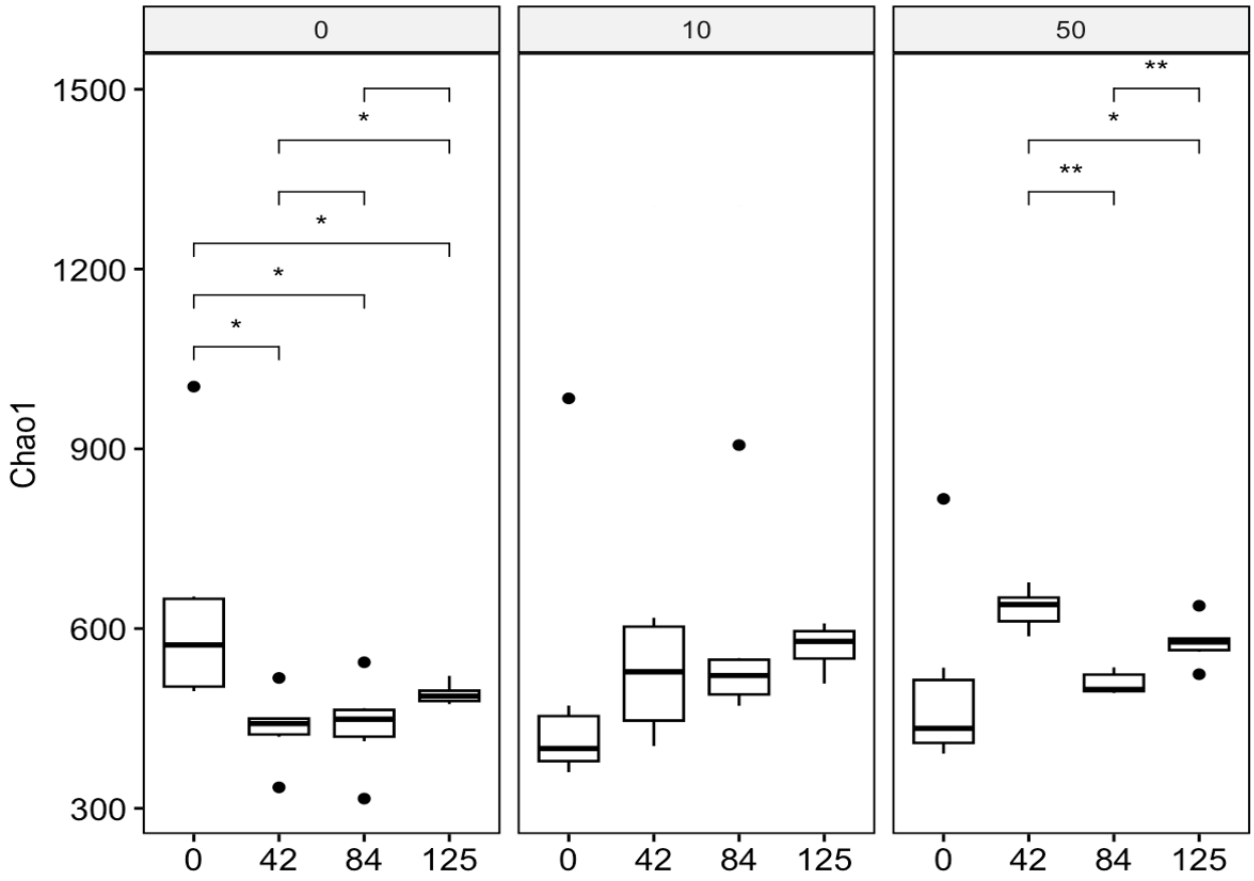

Figure 12. The Alpha diversity analyses based on ASVs using Chao1 indices in samples from different treatment groups on different sampling days. The asterisks indicate that the differences between two points was statistically significant ($P < 0.05$).

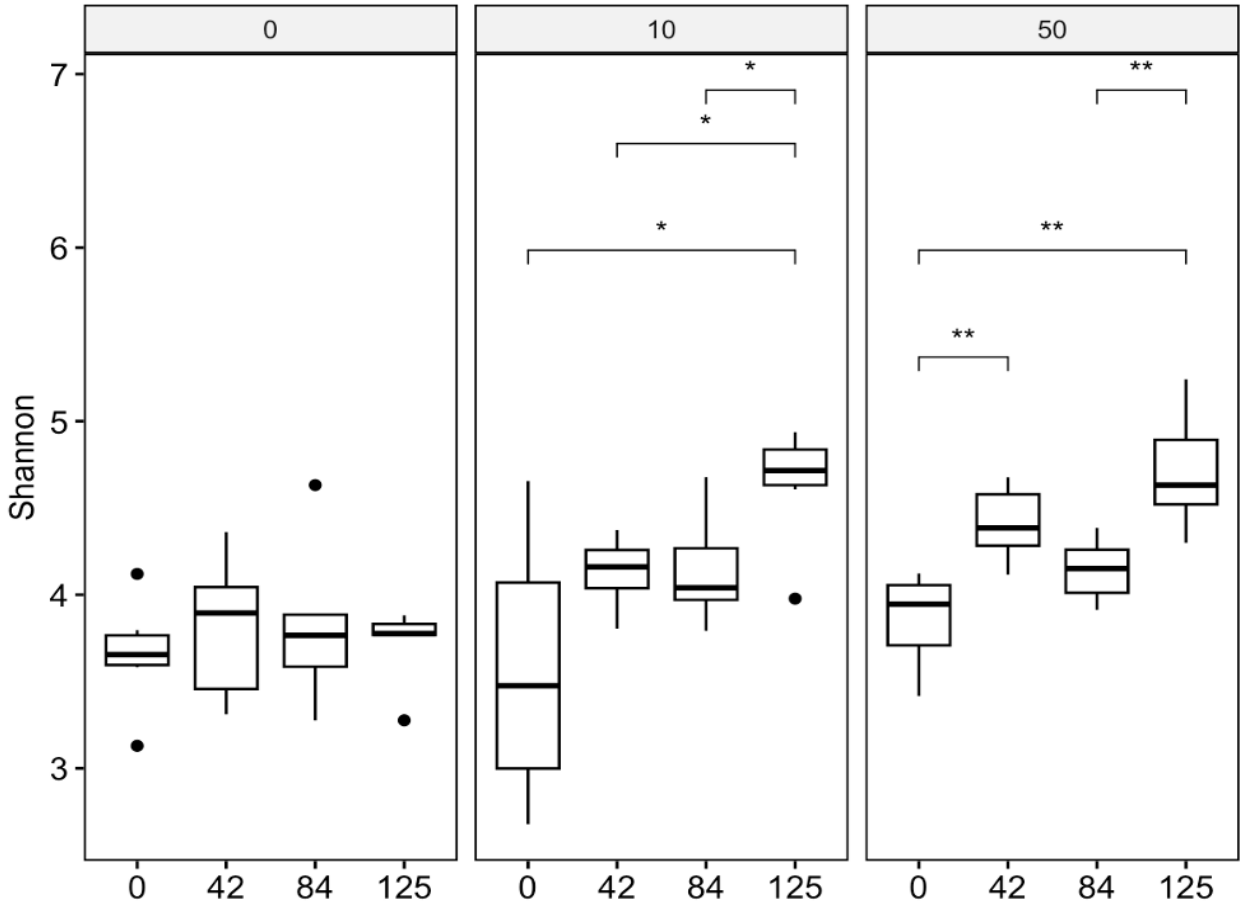

Figure 13. The Alpha diversity analyses based on ASVs using Shannon indices in samples from different treatment groups on different sampling days. The asterisks indicate that the differences between two points was statistically significant.

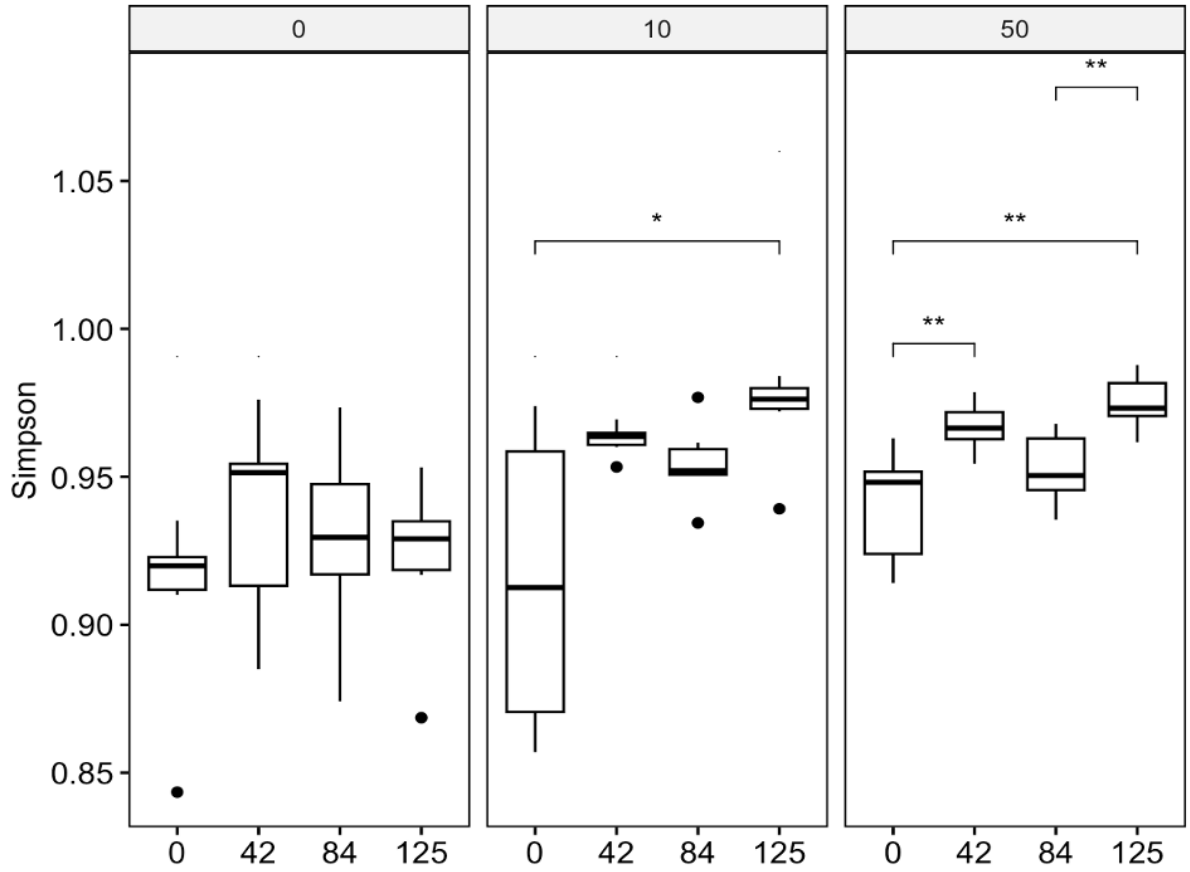

Figure 14. The Alpha diversity analyses based on ASVs using Simpson indices in samples from different treatment groups on different sampling days. The asterisks indicate that the differences between two points was statistically significant.

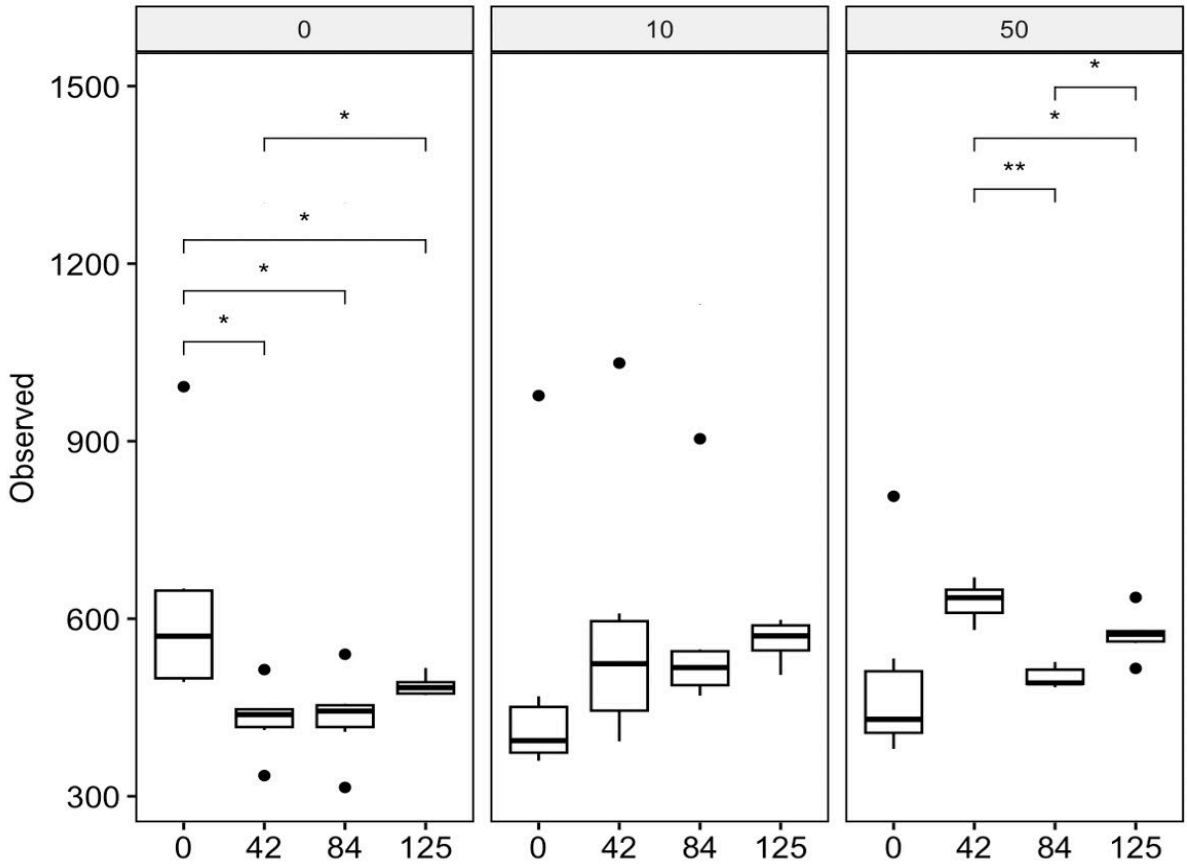

Figure 15. The Alpha diversity analyses using Observed indices in samples from different treatment groups on different sampling days. The asterisks indicate that the differences between two points was statistically significant.

Beta Diversity Analysis

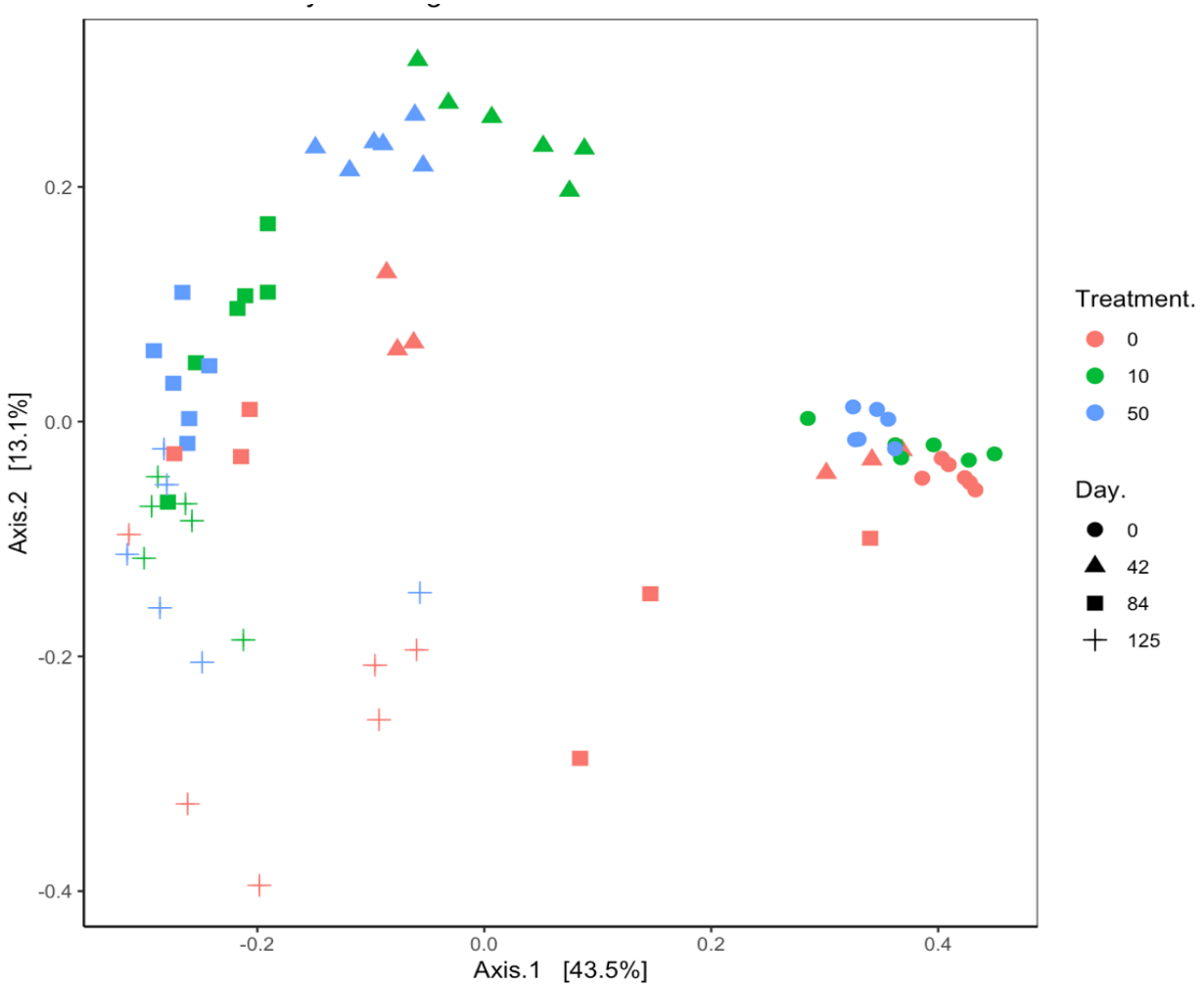

Figure 16. The PCoA (Principal Coordinates Analysis) displays the relationships among microbial communities (at ASV level) from different treatments and sampling days. The axes represent the principal coordinates that account for the most variation in the data. Axis 1 explains 43.5% of the total variation, while Axis 2 explains 13.1%. The colors indicate different treatments, and the shapes represent different time points.

Table 19. Significant Analysis of Similarities of the microbial population (based on genera) between different groups on different days of sampling.

Day/Treatment	**R Statistic**	**P Value**	**Results**
Treatment 10 µg/kg (overall)	0.712	0.02	Significant difference
Treatment 50 µg/kg (overall)	0.556	0.04	Different but with significant overlapping

Table 20. Analysis of Similarities of the microbial population (based on genera) between different groups on different days of sampling.

Day/Treatment	**R Statistic**	**P Value**	**Results**
Day 0	0.102	0.03	No difference
Day 42	0.362	0.08	No difference
Day 84	0.105	0.01	No difference
Last Day	0.383	0.04	No difference
Day 42 to 84	0.363	0.04	No difference
Day 84 to Day 125	0.108	0.03	No difference
Day 42 to Day 125	0.366	0.01	No difference

Table 21. ANOSIM (Analysis of Similarities) of the microbial population based on ASVs between different treatment groups on different sampling days.

Day/Treatment	**R Statistic**	**P Value**	**Result**
Day 0 (10 µg/kg, 50 µg/kg, Control)	0.2305	0.01	No difference
Day 42 (10 µg/kg, 50 µg/kg, Control)	0.5251	0.02	No difference
Day 84 (10 µg/kg, 50 µg/kg, Control)	0.3477	0.02	No difference
Day 125 (10 µg/kg, 50 µg/kg, Control)	0.4379	0.04	No difference

DISCUSSION

Recent studies on MC toxicity have mostly focused on acute exposure, while there is a large research gap in chronic and low dose exposure to MC-LR (Wang et al. 2016). This has been a critical aspect of host health that has been relatively underexplored in the context. In our research, we aimed to investigate the impacts of long-term exposure to low doses of MC-LR on the gut microbiota of mice. We tested the hypothesis that chronic MC-LR exposure induces gradual but notable shifts in the gut microbial community and has a dose-dependent effect on specific bacterial taxa integral to gut health. From the results obtained, we were able to accept the hypothesis, and there was strong evidence found suggesting significant alterations caused by both treatment groups compared to the control.

The increased Shannon diversity index value and observed ASVs were identified only in the treatment group receiving 50 μg/kg MC-LR ($P<0.05$) but not the other samples. These changes could be a result of adaptation, successional changes and/or physiological response of the host to MC-LR (Hu et al. 2008). Further analyses are needed to understand the underlying mechanisms. Moreover, these taxa shifts were only prominent at the end of the study. This was found to be consistent with previous studies that reported the exposure to low dosage of MC-LR significantly increases the species richness and diversity over (Lee et al. 2020, Zhuang et al. 2021). The persistence of these changes in the 50 μg/kg treatment group until the end of the study suggests a dose-dependent response.

From the taxonomic classification, different phyla such as *Actinobacteria* showed significant changes over time that were dependent on the dose of MC-LR. There were changes observed in both doses of MC-LR, however, the extent of changes was different in pattern for each of the treatment groups in same period. The amount of disease susceptibility markers can be

correlated with an increase or reduction in these indicator phyla, such as *Actinobacteria* (Li et al. 2021). Moreover, a significant increase in the abundance of *Firmicutes* was observed in the MC-LR treated groups at the end of the study, along with a decrease in the abundance of *Actinobacteria*, suggesting MC-LR's stimulatory and inhibitory effects, respectively. This aligns with the observation from another study (Lee et al. 2020).

Furthermore, the 10 μg/kg treatment group consistently showed a decrease in the abundance of *Bacteroidetes* and an increase in the abundance of *Firmicutes* over the 125-day experiment compared with the control. Such pattern was absent for the other dose treatment groups, suggesting a treatment-related impact. The 50 μg/kg treatment group also showed changes in the abundance of these phyla, but the changes were less pronounced than those in the 10 μg/kg treatment group, suggesting a dose-dependent effect.

Additionally, the family *Verrucomicrobiaceae* showed a significant decline in abundance in the 10 μg/kg treatment group, and genera such as *Akkermansia, Bifidobacterium, Bacteroides,* and *Clostridium* showed various trends in their abundance across different treatment groups, suggesting specific effects of MC-LR on different bacterial taxa. These are some of the bacterial taxa that play roles in the production of fatty acid that are important to maintain gut homeostasis (Costantini et al. 2017). This result was similar to previous finding from the 8 weeks long study, which showed that SCFA (short-chained fatty acid-producing bacteria) decreased significantly by the end of the experiment (Zhang et al. 2020). However, there was a significant pattern/ fluctuation throughout our study period, which was different from their finding.

In our study, the differential abundance analysis showed that *Bifidobacterium* and *Lactobacillus* were the most depleted genera over time in both treatment groups when compared

to the control group, and more depleted in high treatment group compared to the lower treatment. These two are known to have positive roles in gut homeostasis (Turroni et al. 2014).

The increase in the *Bacilli/Clostridia* ratio has been linked to the activation of inflammatory response against stress response (Pearson-Leary et al. 2019). There was significant decrease in the ratio at the end of the study for the treatment 50 µg/kg compared to day 0 and control. Despite the significant decrease towards the end of the study period, the 50 µg/kg group showed the highest *Bacilli/Clostridia* ratio throughout the experiment, suggesting a potential activation of inflammatory response against MC-LR, this indicates that there could be potential adaptation over time.

High *Bacteroidetes* to *Firmicutes* ratio indicates high susceptibility to obesity (Indiani et al. 2018*).* The 50 µg/kg treatment group showed a sharp increase in the *Bacteroidetes* to *Firmicutes* ratio when compared to both the control and treatment group 10 µg/kg by the end of the study. This suggests that 50 µg/kg treatment has a potential to promote obesity, which is consistent with previous findings (Yang et al. 2023).

Additionally, the PCoA (Figure 16.) at ASV level showed that there were treatment specific alterations forming distinct patterns over time. However, further analysis using the ANOSIM showed no significant difference between the microbial communities of the treatment groups as well as control. ANOSIM was done at the genus level, however, showed significant difference between the samples from treatment 10 µg/kg compared and the other two study groups. This suggests that there was treatment-specific impact on the genus over time, which was more significant in 10 µg/kg compared to 50 µg/kg.

CONCLUSION

In conclusion, this study demonstrated that there were gradual but prominent shift in microbiome communities at low dosage but long-term exposure, supporting our hypothesis.

Increases in *Firmicutes* to *Bacilli* ratio in this long-term study in MC-LR treated gut microbiome suggested possible activation of inflammatory response. Therefore, it can be speculated that chronic inflammation, an imbalance in intestinal homoeostasis, and disruption of the gut barrier could result from long-term exposure to low doses of microcystin-LR. The microbial diversity was found decreased due to the MC-LR exposure. Depletions of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* in the MC-LR treated group indicate that there could be disbalance of gut microbiome due to the exposure to MC-LR.

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CHAPTER IV

SUMMARY

One of the most toxic cyanotoxins produced by cyanobacterial harmful algal blooms is microcystin-LR (MC-LR) (EPA, 2014). MC-LR frequently develops into a persistent environmental contaminant because of its potential to fester in freshwater habitats over the summer (Kochi et al. 2020).

Recently, freshwater pollution caused by CyanoHABs has grown to be a significant issue as they are found in the source of drinking water (Brooks et al. 2016). MC-LR exposure through drinking can disturb the balance in human health as well as that of aquatic animals. Even though the hepatotoxic effects of MC-LR have been thoroughly studied, only a few studies have examined the effects of MC-LR exposure on gut microbiome of animals (Greer et al. 2018; Mills et al. 2021). This research provides insight into disruptions of mouse gut microbiome when exposed to MC-LR based on observations of altered microbial diversity and composition.

The result of this study partially supports the hypothesis that short-term exposure to MC-LR triggers gut dysbiosis. The increase in *Bacteroidetes* to *Firmicutes* ratio, is an indicator of inflammatory response. This inflammatory response was observed in the treatment group 50 μg/kg on day 14 but ceased on the Day 21 of the study. This fluctuating response could be the result of dose dependent or specific response to MC-LR, as the effect is present for 50 μg/kg treatment samples but not for any other study group.

The shift in the population of bacteria producing metabolites like short-chain fatty acids were observed in the MC-LR treated groups. It was found that the long-term exposure of 125 days, which mimics the exposure to MC-LR from CyanoHABs in one warm season, showed depletions of important bacterial taxa such as *Lactobacillus*. This indicates a potential gut disruption by MC-LR that can lead to health implications (digestive disorders).

The change in the host's gut barrier is associated with dysregulated immune homeostasis, which can be inferred by changes in indicator bacterial species such as *Bifidobacterium, Lactobacillus, Actinobacteria*, etc. (Conte et al. 2023). *Firmicutes/Bacteroidetes* (F/B) ratio is widely accepted to indicate normal intestinal homeostasis; and changes in the ratio are associated with obesity and bowel inflammation (Stojanov et al. ,2020). The increase in F/B ratio in the long-term study with 50 μg/kg MC-LR exposure suggests a possible activation of the inflammatory response. Therefore, it can be inferred that long-term exposure to low doses of microcystin-LR could lead to chronic inflammation, disbalance in intestinal homeostasis and disruption of the gut barrier. These effects are not limited to the microbiome but affect the host's overall health, particularly in the context of colorectal health (Yang et al. 2022).

A common dose of MC-LR was used for both of short- and long-term studies (50 μg/kg), which allowed us to examine how the mice's gut microbiome would be affected by MC-LR in different lengths of time. It was found that the microbial community structure was more distinct at the end of the long-term exposure than the short-term exposure.

There were fluctuating patterns in gut microbial taxa abundance for the short-term (21 days) exposure to 50 μg/kg, however the taxa abundance was relatively stable when there the exposure was long-term (125 days). However, there are some caveats associated with this comparsion. Firstly, the starting point of the mice's gut microbiome was different for all the mice,

which makes it tough to draw conclusion for the animals from two completely different studies. Secondly, there were multiple mice in a group, and we calculated their mean difference while looking at the overall change in abundance and diversity (Sarkar et al. 2019). Calculating the mean difference in abundance and diversity across multiple mice in a group can mask individual variations and may not accurately represent the true changes occurring within each mouse. Averages can smooth out the individual differences and may not capture the full complexity of the microbiome dynamics within each mouse (Kodikara et al. 2022; Miyoshi et al. 2018).

Additionally, it is important to understand that the mouse models are employed for exposure research due to their physiological similarities with humans. However, it is essential to consider the limitations of mouse models when translating the results to human. Additionally, for the future direction, it is also essential to understand the effect of MC-LR on both sex of the mice (Mrdjen et al. 2018). The study has a limitation due the fact that the study only uses male mice.

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APPENDIX

SUPPLEMENTAL FIGURES AND TABLES

Table 22. Significant Interactions between treatment groups and sampling time for the Linear Mixed Model Analysis based on the Alpha Diversity Analyses Over Time.

Variable	**Treatment Interaction**	**Day Interaction**	**Estimate**	**p-value**	**Significant**
Shannon Diversity	Control and Treatment 500	Day 0-Day 7	-1.09155	0.01245	Yes
Shannon Diversity	Control and Treatment 500	Day 7-Day 14	1.43810	0.00732	Yes
Simpson Diversity	Overall	Day 7	-0.182881	0.0112	Yes
Simpson Diversity	Treatment 500	Day 7	0.204183	0.0189	Yes

Table 23. ANOSIM (Analysis of Similarities) of the microbial population (based on ASVs) between different treatment groups on different sampling days. There was no significant difference in the microbial populations between the different days of sampling for any of these treatment groups in treatment ($P < 0.05$).

Treatment/Days	**R Statistic**	**P Value**	**Results**
0µg/kg (Day 0, 42, 84, 125)	0.6596	<0.05	No Difference
10 µg/kg (Day 0, 42, 84, 125)	0.8668	<0.05	No Difference
50 µg/kg (Day 0, 42, 84, 125)	0.8259	<0.05	No Difference

Table 24. Distribution of the number of sequences reads annotated at different taxonomic levels for different samples for the short-term microbiome analyses (sample 1-36).

Sample	Codes	Day	Treatment	Phylum Reads	Family Reads	Genus Reads
1	C6A0	0	0	47405	35876	12892
2	C6B0	0	0	92514	59538	59062
3	C8A0	0	0	68697	48416	26344
4	C8B0	0	0	76312	53469	43662
5	L7A0	0	50	86806	60245	48769
6	L9A0	0	50	71559	48060	41595
7	L9B0	0	50	76244	54085	34810
8	L9C0	0	50	76430	58540	43619
9	H5A0	0	500	78863	52961	38276
10	H5B0	0	500	68025	50425	20401
11	H5C0	0	500	73000	47614	23586
12	H4A0	0	500	80477	59526	29932
13	H4B0	0	500	70497	51855	36155
14	C2A7	7	0	84567	60272	25871
15	C8A7	7	0	73234	53637	17999
16	C8B7	7	0	80646	55042	30129
17	C6A7	7	0	86765	62464	34171
18	C6B7	7	0	78458	56370	29364
19	H4A7	7	500	90885	66987	41542
20	H4B7	7	500	90087	65685	31339
21	H4C7	7	500	77314	60281	28175
22	H5A7	7	500	85425	42743	69094
23	H5B7	7	500	85217	55101	46873
24	L7B7	7	50	82003	44548	58239
25	L7C7	7	50	83078	56513	42441
26	L9A7	7	50	85820	53911	36100
27	L9B7	7	50	86620	52009	39298
28	L9C7	7	50	67293	39425	32458
29	C3B14	14	0	77372	40359	37063
30	C8A14	14	0	74819	44014	40263
31	C8B14	14	0	85911	53515	46122
32	C6A14	14	0	21147	12684	9044
33	C6B14	14	0	84264	48221	38829
34	L9A14	14	50	73141	44198	36465
35	L9B14	14	50	83796	53330	39464
36	L9C14	14	50	80795	48880	34750

Table 25. Distribution of the number of sequences reads annotated at different taxonomic levels for different samples for the short-term microbiome analyses (sample 37-72).

Sample	Codes	Day	Treatment	Phylum Reads	Family Reads	Genus Reads
37	L7A14	14	50	109053	88108	37804
38	L7B14	14	50	125680	92516	41732
39	H4A14	14	500	118687	90290	42510
40	H4B14	14	500	108753	63794	61651
41	H4C14	14	500	127419	68630	57473
42	H5A14	14	500	115919	65022	61193
43	H5B14	14	500	137073	105857	39470
44	C2A21	21	0	114985	90824	25384
45	C2B21	21	0	117685	89265	34991
46	C1A21	21	0	138319	86735	42783
47	C8A21	21	0	128918	91031	40031
48	C8B21	21	0	133506	95678	40282
49	L9B21	21	50	141924	98945	45487
50	L9C21	21	50	134290	93289	35103
51	L7A21	21	50	132488	91557	37753
52	L7B21	21	50	131811	98772	28172
53	L7C21	21	50	127424	105123	32659
54	H4B21	21	500	118029	89942	33063
55	H4C21	21	500	123602	103349	28198
56	H5A21	21	500	109886	86997	38196
57	H5B21	21	500	107305	83005	36024
58	H5C21	21	500	122454	92536	43740
59	L9A0	0	500	124628	94660	36107
60	L9C0	0	500	107563	85028	34000
61	C1A7	7	0	113518	80905	53448
62	C3A7	7	0	118057	74008	43384
63	C3B7	7	0	115555	89705	34220
64	H5C7	7	500	107873	76835	39188
65	L7A7	7	50	113716	90915	39869
66	C1A14	14	0	130306	111892	49782
67	C2A14	14	0	129631	89751	56486
68	C2B14	14	0	130966	97373	54029
69	C3A14	14	0	120098	93272	46131
70	H5C14	14	500	115374	91309	35188
71	C3A21	21	0	128389	109762	46771
72	C3B21	21	0	94801	74139	29083

Table 26. Distribution of the number of sequences reads annotated at different taxonomic levels for different samples for the long-term microbiome analyses (sample 1-36).

Sample	Codes	Day	Treatment	Phylum reads	Family reads	Genus reads
1	CA184	84	0	54717	49649	12892
2	CA1L	125	0	97712	92724	59062
3	CA284	84	0	75568	70909	26344
4	CA2L	125	0	80069	76022	43662
5	CA3L	125	0	90924	86463	48769
6	CA384	84	0	78979	73909	41595
7	CB184	84	0	86301	81931	34810
8	CB284	84	0	82872	78955	43619
9	CB384	84	0	85660	77613	38276
10	TC184	84	10	72361	66442	20401
11	TC284	84	10	78644	71117	23586
12	TC384	84	10	83885	79194	29932
13	TD184	84	10	72722	69134	36155
14	TD284	84	10	87443	82196	25871
15	TD384	84	10	75886	70786	17999
16	HE184	84	50	84277	77569	30129
17	HE284	84	50	90962	85383	34171
18	HE384	84	50	80907	75358	29364
19	HF184	84	50	95861	89168	41542
20	HF284	84	50	94182	88234	31339
21	HF384	84	50	81856	77543	28175
22	CB1L	125	0	91927	86920	69094
23	CB2L	125	0	90273	86736	46873
24	CB3L	125	0	87772	82679	58239
25	TC1L	125	10	87421	81652	42441
26	TC2L	125	10	91344	76565	36100
27	TC3L	125	10	91854	81697	39298
28	TD1L	125	10	75754	67222	32458
29	TD2L	125	10	86706	78319	37063
30	TD3L	125	10	84085	73759	40263
31	TE1L	125	50	89836	82617	46122
32	TE2L	125	50	23727	19069	9044
33	TE3L	125	50	90681	82023	38829
34	TF1L	125	50	80474	69333	36465
35	TF2L	125	50	93095	82730	39464
36	TF3L	125	50	85637	75556	34750

Table 27. Distribution of the number of sequences reads annotated at different taxonomic levels for different samples for the long-term microbiome analyses (sample 37-72).

Sample	Codes	Day	Treatment	Phylum reads	Family reads	Genus reads
37	CA142	42	0	120214	114851	37804
38	CA242	42	0	144459	129071	41732
39	CA342	42	0	130687	123093	42510
40	CB142	42	0	126115	115706	61651
41	CB242	42	0	149785	139976	57473
42	CB342	42	0	135556	124577	61193
43	TC142	42	10	146408	135136	39470
44	TC242	42	10	122412	117377	25384
45	TC342	42	10	124157	117428	34991
46	TD142	42	10	143506	120739	42783
47	TD242	42	10	134056	117241	40031
48	TD342	42	10	139404	118337	40282
49	HE242	42	50	147732	132876	45487
50	HE142	42	50	140060	128193	35103
51	HE342	42	50	137322	122351	37753
52	HF142	42	50	141166	123360	28172
53	HF242	42	50	133226	119499	32659
54	HF342	42	50	125206	112037	33063
55	CA10	0	0	137599	133071	28198
56	CA20	0	0	121780	114692	38196
57	CA30	0	0	122335	115976	36024
58	CB10	0	0	136787	129081	43740
59	CB20	0	0	143067	136484	36107
60	CB30	0	0	123136	118313	34000
61	TC10	0	10	123159	115805	53448
62	TC20	0	10	131638	110793	43384
63	TC30	0	10	134081	126620	34220
64	TD10	0	10	122350	112715	39188
65	TD20	0	10	128744	122728	39869
66	TD30	0	10	146834	142182	49782
67	HE10	0	50	143299	136326	56486
68	HE20	0	50	145379	136617	54029
69	HE30	0	50	133388	125638	46131
70	HF10	0	50	129830	114763	35188
71	HF20	0	50	136828	127163	46771
72	HF30	0	50	110613	92429	29083