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The Microbiome of Pediatric and Young Adult Cancer Survivors and Metabolic Syndrome

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Abstract

Background Metabolic syndrome and obesity occur commonly in long-term pediatric cancer survivors and exacerbate other chronic conditions. The intestinal microbiome is associated with metabolic syndrome and obesity in the general population, and is perturbed during cancer therapy. We aimed to determine if long-term survivors of pediatric cancer would have reduced bacterial microbiome diversity, and if these findings would be associated with components of the metabolic syndrome, obesity, and chronic inflammation. Methods We performed a cross-sectional study examining the intestinal microbiome, clinical factors, and biomarkers between 35 long-term survivors and 32 age, sex, and race matched controls. All subjects were ages 10-40, and survivors were at least five years from the time of diagnosis. Results Survivors had decreased alpha diversity compared to controls (Shannon index p=0.001, Simpson index p=0.032) and differently abundant bacterial taxa. Further, among survivors, those who received radiation to the central nervous system or abdomen/pelvis had decreased alpha diversity compared to those that did not receive radiation (Shannon and Simpson $p<0.05$ for both). Although, no specific component of metabolic syndrome or cytokine was associated with measures of alpha diversity, survivors with low adiponectin-lectin ratio, elevated body mass index, and elevated C-Reactive protein had differently abundant taxa compared to those with normal measures. Conclusions The microbiome of cancer survivors remains less diverse than controls even many years after diagnosis, and exposure to radiation may lead to further loss of diversity in survivors. The microbiome may be associated with metabolic syndrome and chronic inflammation in survivors.

The Microbiome of Pediatric and Young Adult Cancer Survivors and Metabolic Syndrome

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Abbreviations:

ASV	Amplicon Sequence Variant
BMT	Blood and Marrow Transplantation
CRP	C-Reactive Protein
ELISA	Enzyme Linked Immunosorbant Assays
REAP-S	Rapid Eating Assessment for Participants

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Data Availability: Data available on request due to privacy/ethical restrictions

Abstract

Background

Metabolic syndrome and obesity occur commonly in long-term pediatric cancer survivors and exacerbate other chronic conditions. The intestinal microbiome is associated with metabolic syndrome and obesity

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in the general population, and is perturbed during cancer therapy. We aimed to determine if long-term survivors of pediatric cancer would have reduced bacterial microbiome diversity, and if these findings would be associated with components of the metabolic syndrome, obesity, and chronic inflammation.

Methods

We performed a cross-sectional study examining the intestinal microbiome, clinical factors, and biomarkers between 35 long-term survivors and 32 age, sex, and race matched controls. All subjects were ages 10-40, and survivors were at least five years from the time of diagnosis.

Results

Survivors had decreased alpha diversity compared to controls (Shannon index $p=0.001$, Simpson index $p=0.032$) and differently abundant bacterial taxa. Further, among survivors, those who received radiation to the central nervous system or abdomen/pelvis had decreased alpha diversity compared to those that did not receive radiation (Shannon and Simpson $p<0.05$ for both). Although, no specific component of metabolic syndrome or cytokine was associated with measures of alpha diversity, survivors with low adiponectin-lectin ratio, elevated body mass index, and elevated C-Reactive protein had differently abundant taxa compared to those with normal measures.

Conclusions

The microbiome of cancer survivors remains less diverse than controls even many years after diagnosis, and exposure to radiation may lead to further loss of diversity in survivors. The microbiome may be associated with metabolic syndrome and chronic inflammation in survivors.

Introduction

Improvements in treatment for childhood cancer patients have led to more than 80% of patients becoming long-term survivors [1], with more than 400,000 survivors of childhood cancer living in the United States [2]. However, among long-term survivors, the rates of chronic severe health conditions remain greatly elevated compared to those of the general population [2-4]. Metabolic syndrome and obesity occur commonly in long-term survivors and exacerbate other chronic health conditions [5-15]. Chronic inflammation has several mechanistic links to obesity and metabolic syndrome [16], and long-term survivors of pediatric malignancies show evidence of chronic inflammation [5,17,18]. How chronic inflammation is mechanistically related to long-term side effects in survivors is still poorly understood.

The microbiome is associated with multiple aspects of pediatric oncology [19]. Active treatment for cancer patients clearly alters the intestinal microbiome [20-22]; however, it is less clear if these changes persist many years after treatment. In otherwise healthy neonates and young children, treatment with antibiotics leads to long-term changes in the microbiome [23-25]. Antibiotic use in infancy is associated with an increased risk of obesity [26], and changes in the microbiome have been associated with several components of metabolic syndrome in the general population [27-29]. These findings have led some to propose that cancer treatment may lead to microbiome reconstitution with pro-inflammatory organisms that increase the risk for metabolic syndrome [8]. We hypothesized that long-term survivors of pediatric cancer and blood and marrow transplantation (BMT) would have reduced bacterial microbiome diversity compared to controls, and these findings would be associated with components of the metabolic syndrome and chronic inflammation. To test this hypothesis, and with the potential to generate new hypotheses, we performed a single-center, cross-sectional exploratory study examining the microbiome, clinical factors, and multiple biomarkers between long-term survivors and age, sex, and race matched controls.

Materials and Methods

Participant Eligibility and Enrollment

Approval was obtained from the Institutional Review Board at the Cleveland Clinic. Survivors were recruited from our comprehensive long-term clinic for survivors of pediatric and young adult cancer and stem cell

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transplantation. Survivors were required to be at least five years from completion of chemotherapy or stem cell infusion. Controls were a convenience sample recruited from members of patient's families, clinic staff and other healthy volunteers. Survivors and controls were required to be between the age of 10 and 40 years at the time of enrollment, and must have refrained from using antibiotics in the preceding six months.

Data and Sample Collection

After consent or assent with parental consent was obtained from survivors and controls, participants filled out a brief history regarding health history, diet, and exercise. Body weight, height, and blood pressure were obtained by clinic staff. Clinical lab testing was performed as indicated for survivors. Serum, plasma, and whole blood were drawn for later analysis for all subjects at study visit. Stool was collected at the patient's home or on-site at the Cleveland Clinic using an Abbexa (Houston, TX) Feces Catcher stool collection kit, then shipped on ice to the Genomics Medicine Biorepository within 30 days. The samples were then aliquoted and centrifuged at 13,200rpm for 15s. The supernatant was removed, and the fecal pellet was stored at -80°C to await further processing.

Clinical Data

Clinical data from survivors were extracted from the electronic medical record. Time from diagnosis was calculated based on the difference from day of enrollment and date of diagnosis. Cyclophosphamide equivalent dose was calculated based on the method of Green, et al [30]. Total days of broad spectrum antibiotics was determined based on the total number of days of the following intravenous agents: ampicillin/sulbactim, aztreonam, cefepime, ceftazidime, ceftriaxone, ciprofloxacin, clindamycin, imipenem, meropenem, Metronidazole, piperacillin/ tazobactam, and vancomycin, received from the time of diagnosis to completion of therapy (or day 100 for transplantation patients).

Biomarker Laboratory Analysis

Lipid and HbA1c measurements were obtained in a Clinical Laboratory Improvement Amendments certified lab, if clinically indicated. If these lab tests were not clinically indicated at the time of the visit, stored samples were then later run on a similar machine at the Cleveland Clinic Laboratory Diagnostic Core (Cobas ce6000 e601 module and c501 module). Additional biomarker assays were also performed at the Cleveland Clinic Laboratory Diagnostic Core. C-Reactive protein (CRP) was measured using the Cobas ce6000 e601 module. The automated Tecan EVO Liquid/Plate System was utilized for for Enzyme Linked Immunosorbant assays (ELISA). ELISA were used to measure Adiponectin, Leptin, Tumor Necrosis Factor alpha, Interleukin-6, and Interleukin-10 (R&D Systems, Minneapolis, MN).

DNA Extraction

Total DNA, including microbial genomic DNA, was extracted from fecal pellets using the QIAmp PowerFecal Pro DNA Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol with minor modifications as follows. Each pellet was resuspended in 800 ul of Solution CD1, after which samples were homogenized with 3 runs of 30 s at 6 m/s in a FastPrep-24 5G bead beater (MP Biomedicals, Solon, OH). Following centrifugation at 15,000 x g for 1 min, the supernatant was transferred to a clean tube and 200 ul of Solution CD2 was added. After another centrifugation at 15,000 x g for 1 min, the supernatant was transferred to a clean tube and mixed with 600 ul of Solution CD3. The resulting lysate was spun through an MB Spin Column and the flow-through discarded. Columns were washed with 500 ul of Solution EA and then 500 ul of Solution C5. Following a drying step at 16,000 x g for 2 min, 50-100 ul of Solution C6 was added to elute the DNA. Buffer-only negative controls were processed identically in parallel. DNA concentrations and quality were determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA), and DNA was stored at -20C until 16S library preparation.

Sequencing and Analysis

16S rRNA gene sequencing methods were adapted from the methods developed for the National Institutes of Health-Human Microbiome Project [31]. Briefly, the 16S rRNA V4 region was amplified and

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sequenced on Illumina Iseq 100 platform using manufacturer's instructions. Raw 16S amplicon sequence (forward reads only) and metadata were demultiplexed using split_libraries_fastq.py script implemented in QIIME1.9.1 [32]. The demultiplexed fastq file was split into sample-specific fastq files using split_sequence_file_on_sample_ids.py script from Qiime1.9.1 [32]. Individual fastq files without nonbiological nucleotides were processed using Divisive Amplicon Denoising Algorithm pipeline [33]. The output of the dada2 pipeline (feature table of amplicon sequence variants) was processed for alpha and beta diversity analysis using phyloseq and microbiomeSeq (http://www.github.com/umerijaz/microbiomeSeq) packages in R [34]. Alpha diversity estimates were measured within group categories using estimate_richness function of the phyloseq package [34]. Nonmultidimensional scaling (NMDS) was performed using Bray-Curtis dissimilarity matrix [35] between groups and visualized by using ggplot2 package [36]. We performed an ANOVA among sample categories while measuring α-diversity using the plot_anova_diversity function in microbiomeSeq package (http://www.github.com/umerijaz/microbiomeSeq). We then performed permutational multivariate ANOVA with 999 permutations to test the statistical significance of the non-multidimensional scaling patterns with the ordination function of the microbiomeSeq package. Pair wise two group analysis was performed using White's non-parametric t-test [37]. We assessed the statistical significance ($P<0.05$) throughout and whenever necessary adjusted P values for multiple comparisons according to the Benjamini and Hochberg method to control false discovery rate [38], while performing multiple testing on taxa abundance according to sample categories.

Additional Statistical Analysis

Metadata, Simpson, and Shannon Index results were exported to Prism (GraphPad Prism Software, La Jolla, CA) for analysis. We tested categorical variables using a two-sided Fisher's exact test. We used an independent two-sample t-test to calculate two-tailed P-value for continuous variables. Pearson correlation was calculated to determine relationships between measures of alpha diversity and clinical data and biomarkers.

Results

Patient Characteristics

A total of 35 long-term cancer survivors and 32 controls were recruited**(Table 1)** . Survivors and controls were frequency matched for age, sex, and race (non-Hispanic White vs. other). Groups demonstrated similar diet types, Rapid Eating Assessment for Participants (REAP-S) score, birth via caesarian section or vaginally, and having been breastfed as an infant. The survivor cohort also underwent chart review to determine cancer type and treatment history, anthropomorphic measurements, clinical laboratory analysis, and additional survey questions (**Table 2**).

Composition of Bacterial Gut Microbiome

Overall, the most common taxa represented among all subjects were*Bacteroides* (19.1%). This was followed by *Blautia*(14.3%), *Faecalibacterium* (7.8%), *Roseburia* (5.8%),*Ruminococcus* (5.8%), *Bifidobacterium* (4.1%),*Fusicatenibacter* (3.8%), *Subdoligranulum* (3.2%),*Akkermansia* (3.1%), and *Anaerostipes* (2.8%). Distribution of major taxa was similar across survivors and controls**(Figure 1)** .

Comparison of Gut Microbiome between Survivors and Controls

Diversity

In order to determine if within-sample diversity (α-diversity) of each group differed, we calculated the Shannon and Simpson diversity indices (which takes into account both evenness and richness of communities) of the samples from survivors and controls. Evaluation of the bacterial microbiome demonstrated reduced alpha diversity ($p<0.05$) utilizing both Shannon and Simpson indices **(Figure 2a).**Bray-Curtis dissimilarity index was used to determine differences in bacterial taxonomic composition between the case and control groups (β-diversity). β-diversity comparisons (**Figure 2b)** between specimens from both groups showed modest differential clustering ($R^2=0.26$, $p=0.006$).

Differently Abundant Taxa

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After adjusting for diet quality (REAP-S score) and type (western, vegetarian, etc.) we compared relative taxa abundance between survivors and controls **(Figure 3).** Eight taxa had statistically significant differential abundance (p<0.05) after adjusting for multiple comparisons (false discovery rate 0.05). Three amplicon sequence variants (ASVs) of *Bacteroides* , one ASV of*Lachnospiraceae* , and one ASV of *Holdemanella* were more common in controls, suggesting these taxa may be depleted with cancer therapies, whereas two ASVs of *Lactobacillus* , and one of*Facealitalea* were more common in survivors.

Microbiome among Survivors

We next analyzed features of the microbiome among survivors based on previous exposures that we hypothesized to cause long-term changes in the microbiome. Alpha diversity differed between the type of disease survivors experienced (hematologic malignancies, central nervous system tumors, other solid tumors, or bone marrow transplantation for a non-malignant condition), but smaller group sizes limited ability to make definitive comparisons **(Supplementary Figure 1).** We did not observe a correlation between the time from diagnosis to study enrollment and the Shannon index (R=0.04, p=0.80).

To measure overall chemotherapy intensity, cyclophosphamide equivalent dosing was calculated for each survivor [30]. We did not observe a correlation between cyclophosphamide equivalent dose and Shannon index (R=0.03, p=0.89). In contrast, we did detect several differently abundant taxa between those who received above and below the mean cyclophosphamide equivalent dose **(Supplementary Figure 2a).**

We next determined the role of antibiotic intensity on the microbiome in survivors by calculating the total days of intravenous broad spectrum antibiotics received during the treatment period. We did not observe a correlation between total days of broad spectrum antibiotics and Shannon index (R=0.06, p=0.81). However, we did observe several differently abundant taxa between those who received above and below the mean antibiotic days **(Supplementary Figure 2b).**

We also examined the role of radiation therapy on the microbiome in the survivor cohort. Survivors who had radiation exposure to the abdomen or pelvis were compared to those survivors who did not receive radiation to these locations. For those that received radiation to the abdomen/pelvis, α-diversity as measured by Shannon and Simpson indices was reduced compared to those that did not (p<0.05 for both)**(Figure 4a).** Further, differently abundant taxa were seen between these two groups, with those having received radiation to the abdomen/pelvis demonstrating a greater proportion of several*Bifidobacterium* ASVs whereas those that did not receive radiation had a greater abundance of several *Blautia* ASVs **(Figure 4c).** We also analyzed the impact of radiation exposure to the central nervous system and found that those that received central nervous system radiation had decreased α-diversity as measured by Shannon and Simpson index **(Figure 4b).**

Relationship between the Microbiome, Metabolic Syndrome, and Chronic Inflammation

Next we compared features of the microbiome and subject clinical characteristics, including components of metabolic syndrome as well as several markers of inflammation, amongst survivors. We did not observe a statistically significant correlation with either Shannon or Simpson Index and Hemoglobin A1c, High Density Lipoprotein, Low Density Lipoprotein, Adiponectin- Leptin ratio, systolic blood pressure, diastolic blood pressure, or body mass index **(Supplementary Table 1).** We also did not observe a significant correlation between Shannon or Simpson index and levels of C-Reactive protein, Tumor Necrosis Factor alpha, or Interleukin-10 **(Supplementary Table 2).** A modest correlation was seen between Simpson index and levels of Interleukin-6 amongst survivors (R=-0.41, P=0.02), but no correlation was seen between Shannon index and Interleukin-6 levels (R=-0.04, P=0.81).

We also examined differently abundant taxa in subjects based on adiposity, body mass index and inflammation. Subjects with less favorable Adiponectin-Leptin ratio (<1.0) had increased abundance of multiple *Bacteroides* ASVs (**Supplemental Figure 3)** . Survivors with differing body mass index and C-Reactive protein levels also demonstrated differently abundant taxa after adjusting for a FDR of 0.05 **(Supplemental Figure 4, Supplemental Figure 5)** .

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Discussion

Overall, we found that long-term survivors of childhood cancer have decreased gut bacterial microbiome diversity and differently abundant bacterial taxa, even years after the completion of therapy. Further, these changes are apparent despite the finding of similar present diet practices between survivors and controls. Additionally, radiation therapy, particularly to the abdomen and pelvis, but also cranial radiotherapy, appears to be associated with reductions in gut microbial diversity and differently abundant taxa. Interestingly, exposures such as chemotherapy and antibiotic intensity were not directly associated with alpha diversity in survivors, though they were associated with differentially abundant taxa. In this study, alpha diversity itself did not appear to be associated with components of the metabolic syndrome or chronic inflammation among childhood cancer survivors, but low prevalence of metabolic derangements in the study population may have precluded this finding. Notably, however, among survivors with increased adiposity, elevated body mass index, and increased inflammation, differently abundant taxa were present, suggesting a potential interaction with the microbiome.

Two previous studies in similar populations also demonstrated reduced microbial diversity in childhood cancer survivors. Among 13 long-term Hodgkin lymphoma survivors and twin controls (8 dizygotic, 5 monozygotic), survivors had fewer operational taxonomic units in fecal samples compared to their co-twin control [39]. A recent study of adult survivors of childhood acute lymphoblastic leukemia from Malaysia compared survivors to healthy controls [18]. Similar to the present study, Chua, et al. recruited controls among healthcare works, siblings, and other volunteers and were matched for sex and ethnicity, though neither subjects nor controls were excluded for recent antibiotic use. Chua, et al. also found that survivors had altered composition of taxa and decreased alpha diversity of bacteria compared to healthy controls, but used anal swabs for sample acquisition.

Survivors of childhood acute lymphoblastic leukemia have been previously demonstrated to have increased levels of inflammation compared to controls [17]. Indeed, Chua, et al. demonstrated modest associations between bacterial taxa and Interleukin-6 and C-Reactive protein, though the role of cancer history, chemotherapy and antibiotics in these associations was unclear [18]. In the present study we were not able to detect an association between alpha diversity and markers of chronic inflammation in survivors, but did see differences in taxa abundance based on levels of C-Reactive protein. As opposed to the work previously performed, this is the first study to clearly demonstrate alterations in microbiota in a heterogeneous cohort of long-term survivors based on multiple different underlying malignancies. Additionally, although radiation has been shown to lead to short term changes in the microbiome in oncology patients [40,41], ours is the first study to demonstrate a relationship between radiation and microbiome diversity many years after treatment.

Our study has some limitations. First, our cohort did not have high levels of phenotypic abnormalities. Hyperlipidemia and insulin resistance were uncommon, and most patients did not possess components of the metabolic syndrome. This limited our ability to detect an association of the microbiome with these late effects, and perhaps a larger or older cohort of patients may have improved that power. Further, the associations between differently abundant taxa and phenotypes demonstrated in this study do not necessarily imply causation, and mechanistic studies would be required to further characterize these relationships. Additionally, we did not perform internal transcribed spacer sequencing in this study to detect a potential interplay between the bacterial microbiome and the gut fungal microbiome, which has been implicated in obesity and other diseases [42,43]. Finally, this was a single institution exploratory study, and microbiome findings may be unique to the patients seen in our clinic or geography. Regardless, this study still defines important relationships ripe for further exploration.

Overall, this study demonstrates that long-term survivors of childhood cancer have decreased gut bacterial microbiome diversity and differently abundant bacterial taxa, even years after the completion of therapy. Additionally, radiation may be particularly important in microbial diversity among this cohort. In future experiments, we aim to further categorize the metabalome in a similar cohort of patients to better understand if the difference found on 16S sequencing results in alterations of metabolic products in the blood-stream. We also aim to explore potential relationships with the fungal microbiome and gain a better understanding

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of mechanisms via gnotobiotic mouse experiments. Further, in order to detect associations with clinically relevant late effects, we aim to perform a larger multicenter study using older subjects with greater prevalence of comorbidities.

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Tables

Table 1. Cases v. Controls

	Survivors (N=35)	**Controls (N=32)**	**P-Value**
Age at Recruitment (mean, SE)	22.2 (1.03)	24.7 (0.94)	0.08
Sex (% Female)	45.7%	53.1%	0.63
Race (% Non-Hispanic White)	74.3%	53.1%	0.08
Diet Type (% American/ Western)	77.1%	71.9%	0.78
REAP-S Score (mean, SE)	28.1 (0.78)	29.2 (0.91)	0.36
Born via Vaginal Delivery (%)	69.0%	74.1%	0.77
Breastfed as Infant (%)	66.7%	79.2%	0.36

Table 1. SE: standard error; REAP-S: Rapid Eating Assessment for Participants

Table 2 Characteristics of Cancer Survivor Cohort (N=35)

Age at Diagnosis (years; median, range)
Time Since Diagnosis (years; median, range)
Disease Type Hematologic Malignancy Central Nervous System Tumor Other Solid Tumor BMT for Non-Malignant Cond
Radiation Central Nervous System Radiation Abdomen/Pelvis Radiation
Cyclophosphamide Equivalent Dose (median, range)
Underwent BMT Allogeneic Autologous
Received Broad Spectrum Antibiotics During Therapy (%) Yes No Unavailable

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Total Days of Broad Spectrum Antibiotics (median, range) **Measurement** (median, range) Hemoglobin A1c Low-De

Table 2. Primary Diagnosis include: Acute Lymphoblastic Leukemia (12), Hodgkin Lymphoma (3), Medulloblastoma (2), Osteosarcoma (2) Non-Hodgkin Lymphoma (2) Neuroblastoma (2), Low Grade Glioma (2), Wilms Tumor (2), Acute Myeloid Leukemia (1), Anaplastic Oligoastrocytoma (1), Ewing Sarcoma (1), Langerhans Cell Histiocytosis (1), Mixoid Fibrous Histiocytoma (1), Rhabdomyosarcoma (1), Primitive Neuro-Ectodermal Tumor (1), Other (1).

Figures

Figure 1. Genus-level abundance in controls and survivors

Figure 2. α- and β-diversity of bacterial communities in survivors and controls. (A) α-diversity based on Shannon and Simpson index (B) β-diversity displayed as principal coordinate analysis based on Bray-Curtis dissimilarity index.

Figure 3. Differential abundance analysis of survivors (orange) versus controls (pink). Listed taxa were statistically significantly different in abundance, after adjusting for multiple comparisons.

Figure 4. (A) Alpha diversity based on having received radiotherapy to the abdomen/pelvis, and (B) by receipt of radiotherapy to the central nervous system. (C) Differently abundant taxa based on radiation to abdomen/ pelvis.

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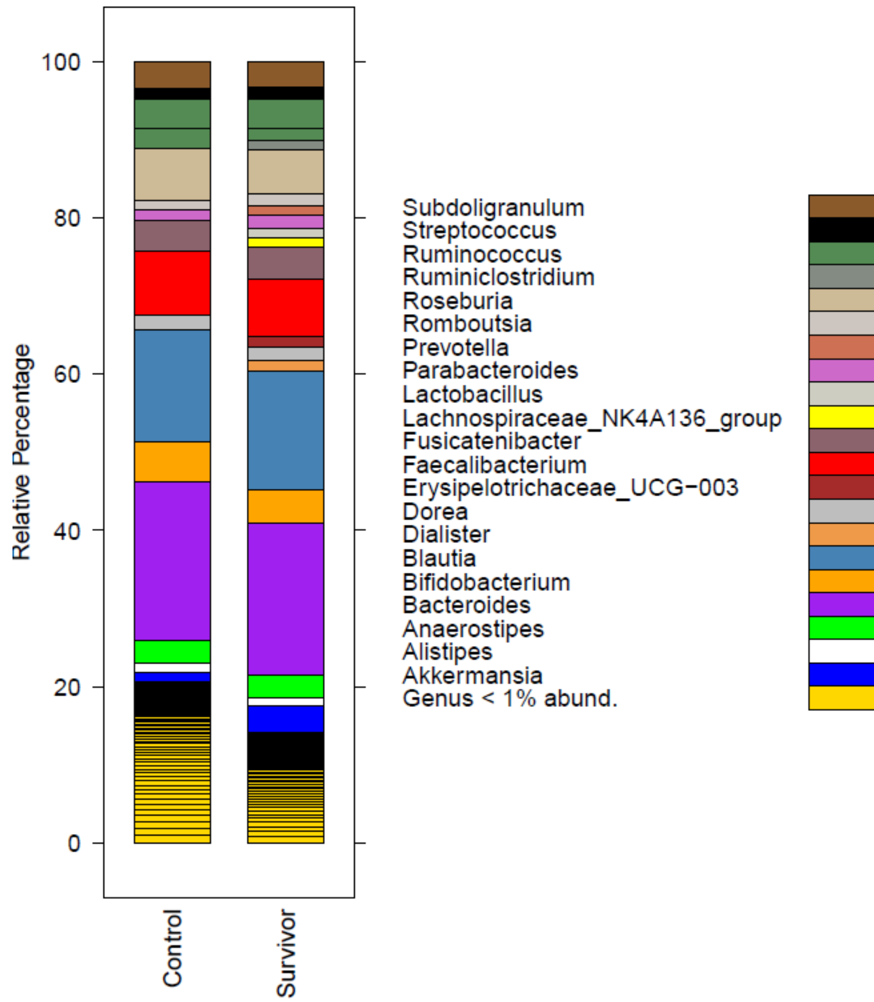

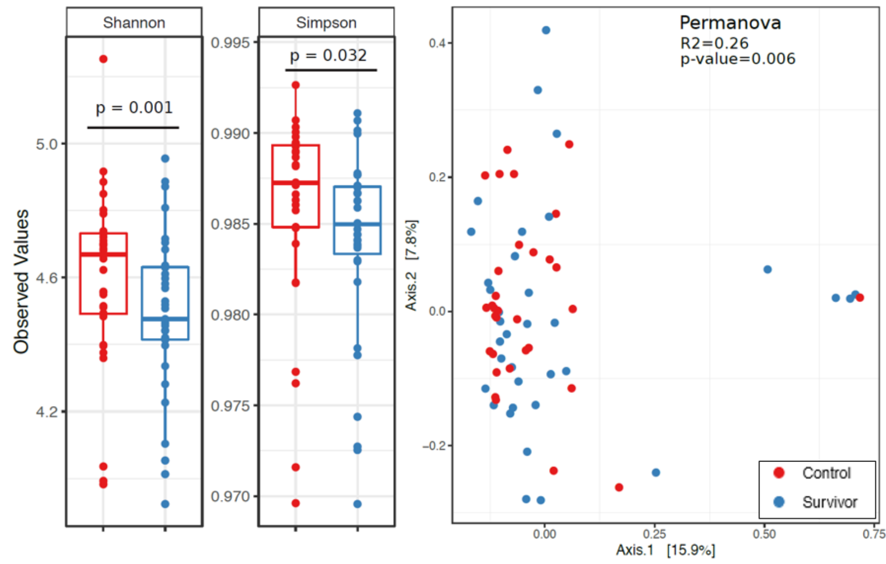

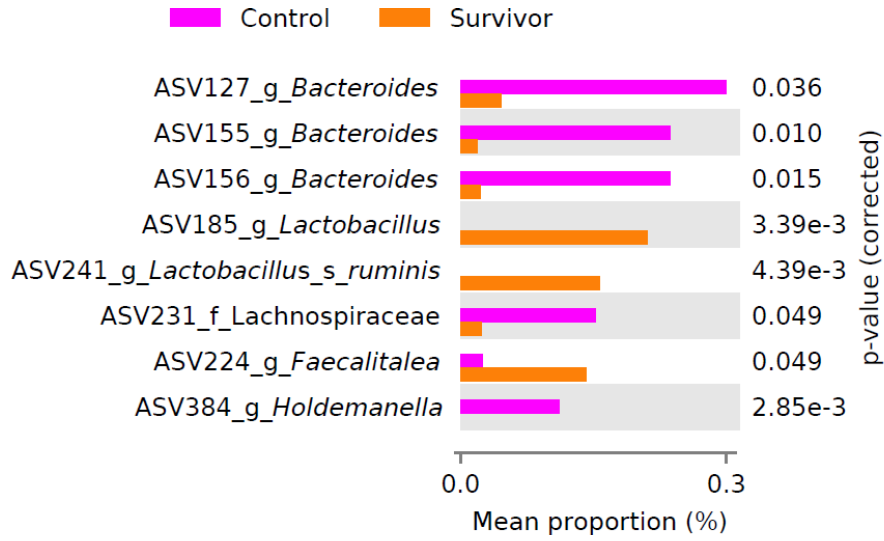

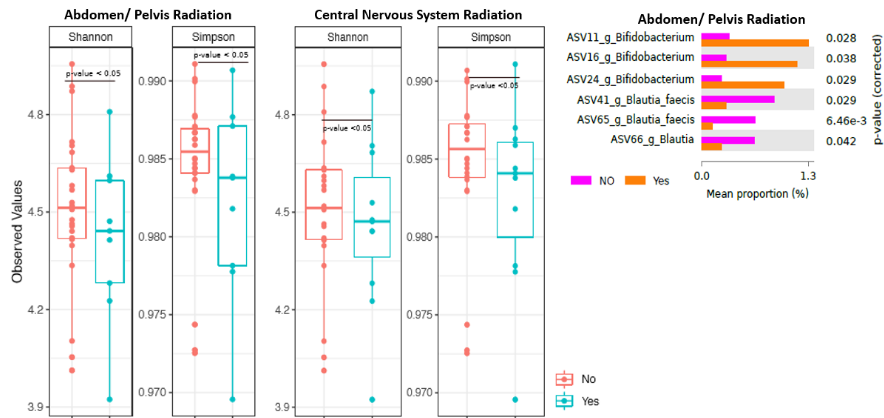

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