The role of Gut Microbiome in Anorexia Nervosa

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One sentence description

Gut microbiome alterations modulate hypothalamic gene expression and microbial diversity in mouse models of anorexia, providing insights into neuropeptide regulation mechanisms.

Key words

Gut microbiome, Eating disorders, Activity-based anorexia model, Appetite regulation, Neuropeptide Y, Gut barrier

ABSTRACT

The gut microbiome has a profound impact on neuropsychiatric disorders and is hypothesized to influence neuropeptides associated with appetite regulation and perception. In this project, we aim to investigate the impact of microbiome alterations on hypothalamic gene expression and microbial diversity. We perform bioinformatic analyses on 16S rRNA sequencing data following an activity-based anorexia (ABA) mouse model experiment [1] to explore microbial and gene expression changes in antibiotic-treated, germ-free, and control mice. The associated experiment samples' sequencing data were downloaded from the SRA (PRJEB70936) and processed on the UMD bioi605 cluster using a reproducible DADA2- pipeline. This included quality score assessment, trimming, dereplication, denoising, chimera removal, and taxonomic assignment using the SILVA 138.2 database. This manuscript outlines the initial data processing and analysis steps in preparation for downstream statistical and biological interpretation.

INTRODUCTION

Anorexia nervosa (AN) is a complex psychiatric disorder marked by extreme food restriction, low body weight, and an intense fear of gaining weight, often accompanied by excessive exercise and a distorted body image. While traditionally viewed as a behavioral condition, recent research highlights the potential role of systemic inflammation and altered gut microbiota in its pathophysiology, as AN patients exhibit signs of chronic low-grade inflammation, leading to deficits in intestinal permeability. [2]

Evidence suggests that the gut-brain axis, particularly microbiome-mediated modulation of hypothalamic appetite regulation, may contribute to the onset and persistence of AN. To explore this, this study uses a mouse model combining food restriction and hyperactivity to investigate the influence of the gut microbiome (specifically the expression levels of eight hypothalamic appetite-regulating peptides and two reference genes) on the onset and progression of AN.

The research studies three groups of mice: control (healthy gut microbiome), antibiotic-treated, and germ-free (with no microbiome). Mice were further divided based on diet and access to a running wheel across three experimental phases.

The groups of mice are summarized in the table below:

	Fed ad libitum (AL)	**Fed restrictively (FR)**
Access to running wheel	Access, AL	Access, FR
No access to running wheel	No access, AL	No access, FR

Fecal samples from all the groups were collected at the end of each phase, genomic DNA was isolated and used to construct bacterial 16S rRNA amplicon sequencing libraries. As part of the gene expression analysis, six appetite-regulating neuropeptides, two intestinal tight junction proteins and two mucin protein family members were studied.

The supplementary table from the original study paper lists the primers studied.

Supplementary Table 1: List of primers used for gene expression analysis in mouse hypothalamus and intestine.

Gene	**Forward Primer (5´→ 3´)**	**Reverse Primer (3´→ 5´)**	**Amplicon length (bp)**
Hypothalamus			
Hprt	GGTTAAGCAGTACAGCCCCA	GGCCTGTATCCAACACTTCG	81

Actb	TTGCTGACAGGATGCAGAAG	GTACTTGCGCTCAGGAGGAG	86
NPY	CTGCGACACTACATCAATCT	CTTCAAGCCTTGTTCTGG	124
AgRP	TGCAGACCGAGCAGAAGAAG	GACTCGTGCAGCCTTACACA	114
MCH	AGCATCAAACTAAGGATGGCA	GGCGACAACGGATCTTTCTG	195
Orexin	ACCGTAACTACCACCGCTTT	GGGGGAAGTTTGGATCAGGA	86
CRH	GAAAATGTGGCCCCAAGGAG	AGCCACCCCTCAAGAATGAA	106
TRH	TCTGCAGAGTCTCCACCTTG	CGAAGATCAAAGCCAGAGCC	73
CART	GCTGCTACTGCTACCTTTGC	TTCGATCAGCTCCTTCTCGT	118
POMC	CCATAGATGTGTGGAGCTGGTG	CATCTCCGTTGCCAGGAAACAC	365
Intestine			
Hprt	GGTTAAGCAGTACAGCCCCA	GGCCTGTATCCAACACTTCG	81
CasC3	TTCGAGGTGTGCCTAACCA	GCTTAGCTCGACCACTCTGG	96
ZO-1	CCACCTCTGTCCAGCTCTTC	CACCGGAGTGATGGTTTTCT	249
OCCLU	actcctccaatggacAaagtg	ccccacctgtcgtgtagtct	249b
MUC2	GATGGCACCTACCTCGTTGT	GTCCTGGCACTTGTTGGAAT	246
MUC13	GAGGAGAAACAGGAGCATAG	GGACATCAAGGGAGAAG	107

Hprt - Hypoxanthine phospho-ribosyltransferase 1; ActB - Actin beta; NPY – Neuropeptide Y; AgRP – Agouti-related peptide; MCH – Melanin concentrating hormone; CRH - Corticotropin Releasing Hormone; TRH - Thyrotropin Releasing Hormone; CART - Cocaine- and amphetamine-regulated transcript; POMC – pro-opiomelanocortin; CasC3 - Cancer susceptibility candidate 3; ZO-1 – zonula occludens-1; OCCLU – occludin ; MUC2 – mucin 2; MUC13 – mucin 13

MATERIALS AND METHODS

Study Design

Fecal pellets from control and ATB-treated mice were collected at four time points throughout the experiment to assess microbiome dynamics. Samples were collected at baseline, post-antibiotic treatment, post-acclimation, and at study termination. Analysis focused on shifts in alpha and beta diversity, compositional changes at the phylum level, and dispersion metrics between treatment groups.

Data Acquisition

Raw sequencing data were obtained from the Sequence Read Archive (SRA) under BioProject accession **PREJEB70936,** corresponding to the study by Roubalova et al. (2024), which investigated the gut microbiome's role in appetite regulation in mouse models of anorexia nervosa. Metadata associated with this project were downloaded using the *cyoa* toolkit on the UMD bioi605 cluster. The accession metadata CSV file was parsed and used to link sequence files to experimental conditions.

To obtain the data, we first configured the computing environment using the following commands:

- module add sra
- vdb-config –interactive
- module add perl
- module and cyoa
- cyoa –method sradownload –input PREJEB70936

The Mus Musculus reference genome (GREm38), annotated by Ensembl was downloaded from here and is intended to be used for differential expression analysis of the above listed neuropeptides across the three experiment groups.

Metadata Processing

The project's metadata (PRJEB70936_sra.csv) was downloaded from NCBI and imported into R, and processed using the *readr*, *dplyr*, and *stringr* packages. Key variables, including mouse identifiers, treatment condition - *control* (CTRL) and *anti-biotic treatment* (ATB), and experimental time points were extracted from the protocol field. Cleaned metadata were saved in *mouse_label_data.csv* and linked to FASTQ files for downstream analysis.

Quality Control and Trimming

Raw paired-end FASTQ reads were assessed using the *plotQualityProfile()* function in the *DADA2* package. Based on quality score visualization and reference scipts from the study [3], truncation lengths of 270 bp for forward reads and 190 bp for reverse reads were selected. Reads were then trimmed and filtered using the *filterAndTrim()* function with default parameters, removing low-quality bases and reads with ambiguous nucleotides.

Denoising, Merging, and Chimera Removal

Filtered reads were dereplicated and denoised using the *dada()* function with *selfConsist=TRUE* to allow joint estimation of error profiles and sample composition. Paired reads were then merged using *mergePairs()*, and amplicon sequence variants (ASVs) were compiled into a count matrix using *makeSequenceTable()*. Chimeric sequences were identified and removed using the *removeBimeraDenovo()* function in DADA2 using the *consensus* method. The resulting non-chimeric ASV table represented high-confidence biological variants across all samples.

Taxonomic Assignment and Phyloseq Object Construction

Amplicon sequence variants (ASVs) were classified taxonomically using the *assignTaxonomy()* function in the DADA2 package, with the *SILVA v138.2* reference database and a minimum bootstrap confidence of 80. The resulting ASV table, taxonomic assignments, and haplotype sequences (haplo.fasta) were merged into a unified *phyloseq* object. Sample metadata were formatted and added using *sample_data()* to enable grouping by treatment condition and time point.

Normalization and Rarefaction

To prepare for diversity analysis, two normalization approaches were applied. First, the dataset was converted to relative abundances using transform_sample_counts() in phyloseq. Second, to control for uneven sequencing depth across samples, rarefaction was performed using rarefy_even_depth() with a fixed random seed for reproducibility.

Taxonomic Composition Visualization

Relative abundance data were aggregated at the phylum level using tax_glom() and transformed to long format using psmelt(). To focus on dominant taxa, only the top eight phyla by total abundance were retained; all others were grouped as "Other." The resulting data were used to generate stacked barplots, grouped by mouse and timepoint, using ggplot2.

RESULTS

Visualizing The Quality

Examining the forward read quality plots, we observed that the scores remain high and stable up to approximately cycle 240–250, after which the read quality began to decline. The quality scores for the forward read of two samples, SATB22_F_trus2R and SATB23_F_trus2R are shown in Figure 1 below.

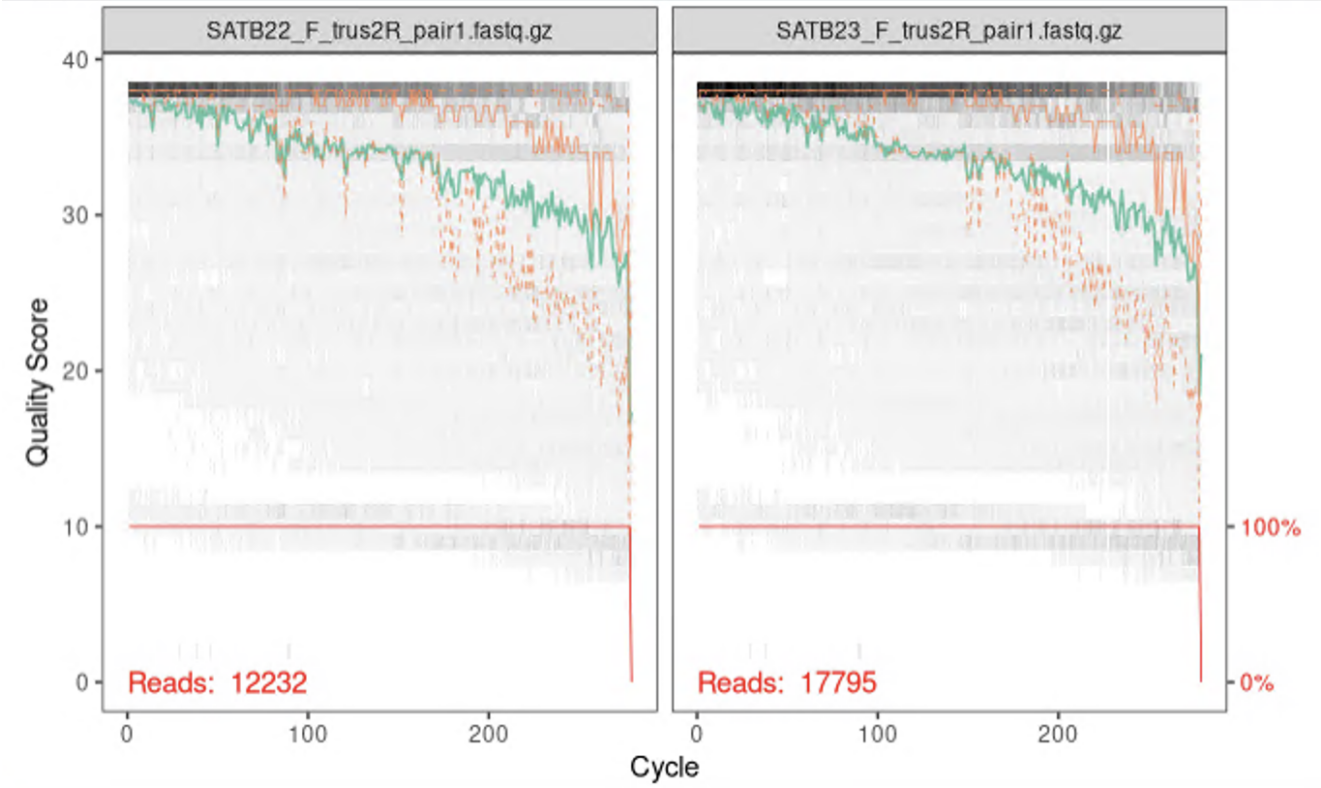

Figure 1. Forward read quality plot

The reverse read quality starts to decline after cycle 180. The quality scores for the reverse read of two samples, SATB22_F_trus2R and SATB23_F_trus2R are shown in the plot below *(Figure 2).*

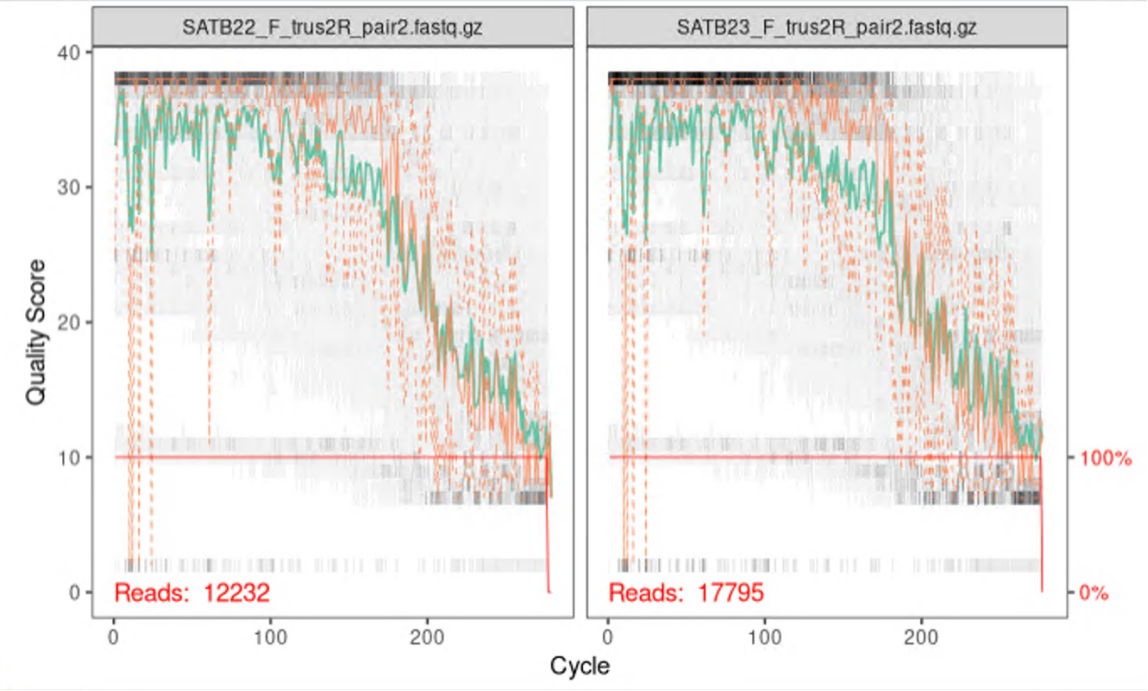

Figure 2. Reverse read quality plot

Taxonomic Composition Bar plot

We investigated the differences in microbial composition across the ATB and CTRL groups and found that antibiotic exposure disrupted the normal microbial balance *(Figure 3).* Several bacterial phyla that were nearly absent in the CTRL groups, such as Pseudomonadota (blue) and Verrucomicrobiota (pink), appeared in the ATB group.

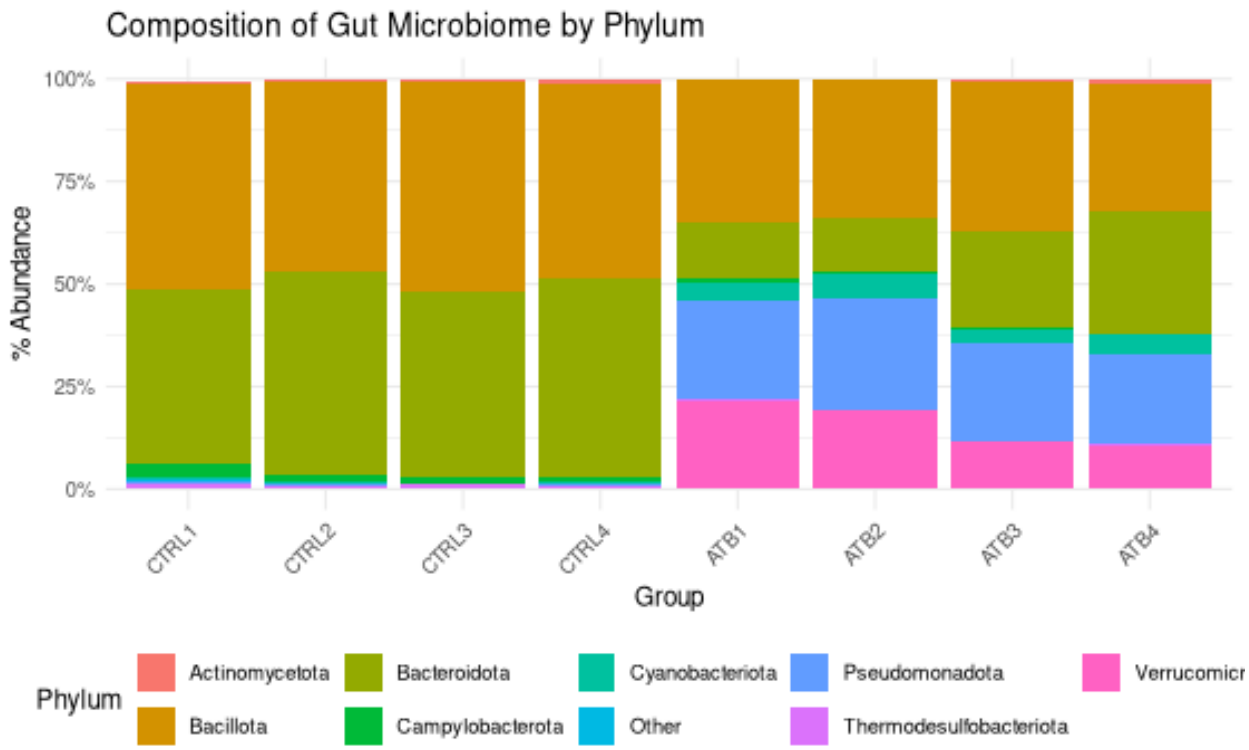

Figure 3. Taxonomic Composition boxplot, which looks at the average gut microbiome composition (at the phylum level) for a specific group of mice

Alpha Diversity Boxplot

To assess within-sample microbial diversity, we calculated three alpha diversity metrics: **Chao1, Observed ASVs,** and **Shannon index**. These metrics reflect species richness and evenness across experimental groups. The resulting boxplots *(Figure 4)* revealed that gut microbial alpha diversity was significantly reduced in mice treated with antibiotics (ATB1–ATB4) compared to control groups (CTRL1–CTRL4).

- Both **Chao1***(Figure 4a)* and **Observed ASVs** *(Figure 4b)* showed a clear reduction in richness following antibiotic treatment.
- **Shannon diversity** (*Figure 4c),* which reflects both richness and evenness, declined particularly in the ATB2 and ATB3 groups, indicating substantial disruption to the microbial ecosystem.
- Notably, **ATB1**, representing the earliest post-treatment timepoint, displayed diversity levels like control mice, suggesting the antibiotics had not yet taken effect.
- By **ATB4**, a slight increase in all three metrics was observed, indicating partial recovery of the gut microbiome.

These patterns underscore the temporal impact of antibiotic exposure on microbial communities and support the use of these metrics as sensitive indicators of gut dysbiosis and recovery.

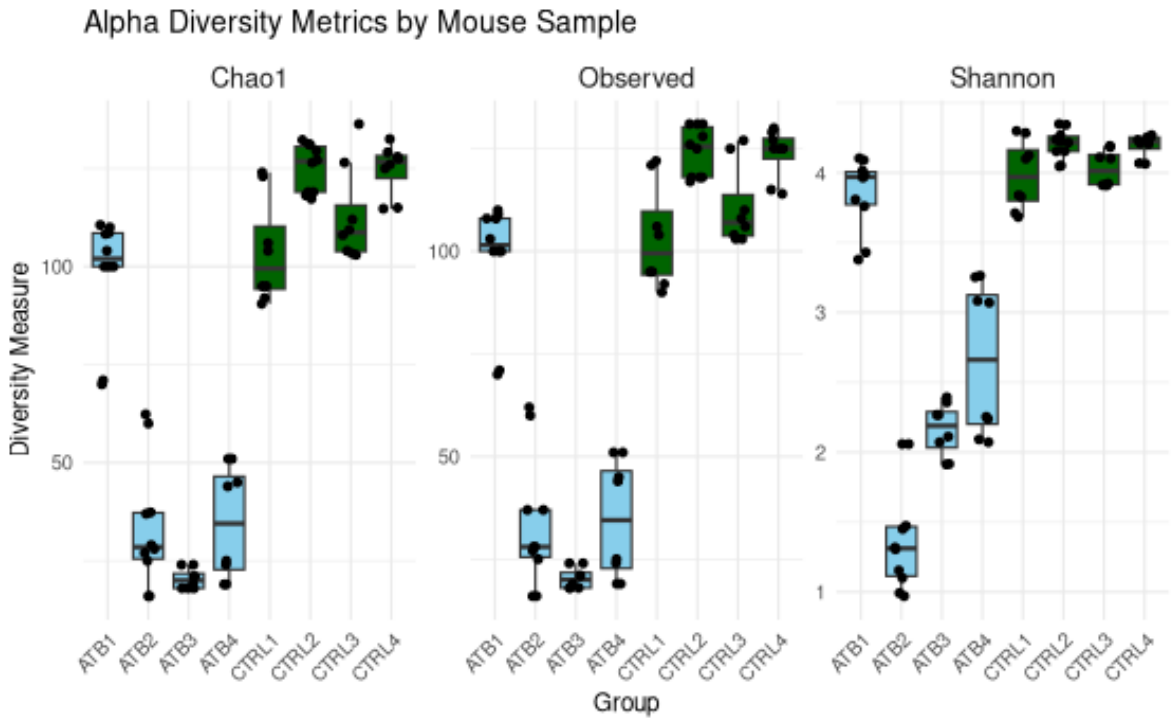

Figure 4. Visualization of the alpha diversity metrics. Chao1 box plot (a) estimated total richness including rare taxa. Observed box plot (b) the count of the unique taxa. Shannon box plot (c) richness and evenness how evenly taxa are distributed.

Beta Diversity Plot

To assess between-sample differences in gut microbiome composition, a Principal Coordinates Analysis (PCoA) was performed using Bray-Curtis distance metrics. Each point in the ordination plot represents the microbial community of an individual mouse at a specific timepoint *(Figure 5).* The first principal coordinate (Axis 1) accounts for 37.1% of the total variation, while the second principal coordinate (Axis 2) explains 22.4%.

Control (CTRL) mice clustered closely together, indicating a high degree of similarity in their microbiome composition over time. In contrast, antibiotic-treated (ATB) mice were more widely dispersed and formed distinct clusters separate from the CTRL group, suggesting that antibiotic exposure created a significant impact on microbial community structure. Likewise, ATB1 samples clustered nearer to the CTRL group, reflecting their

collection before antibiotic administration. These results highlight the substantial impact of antibiotic treatment on microbiome composition and its progression over time.

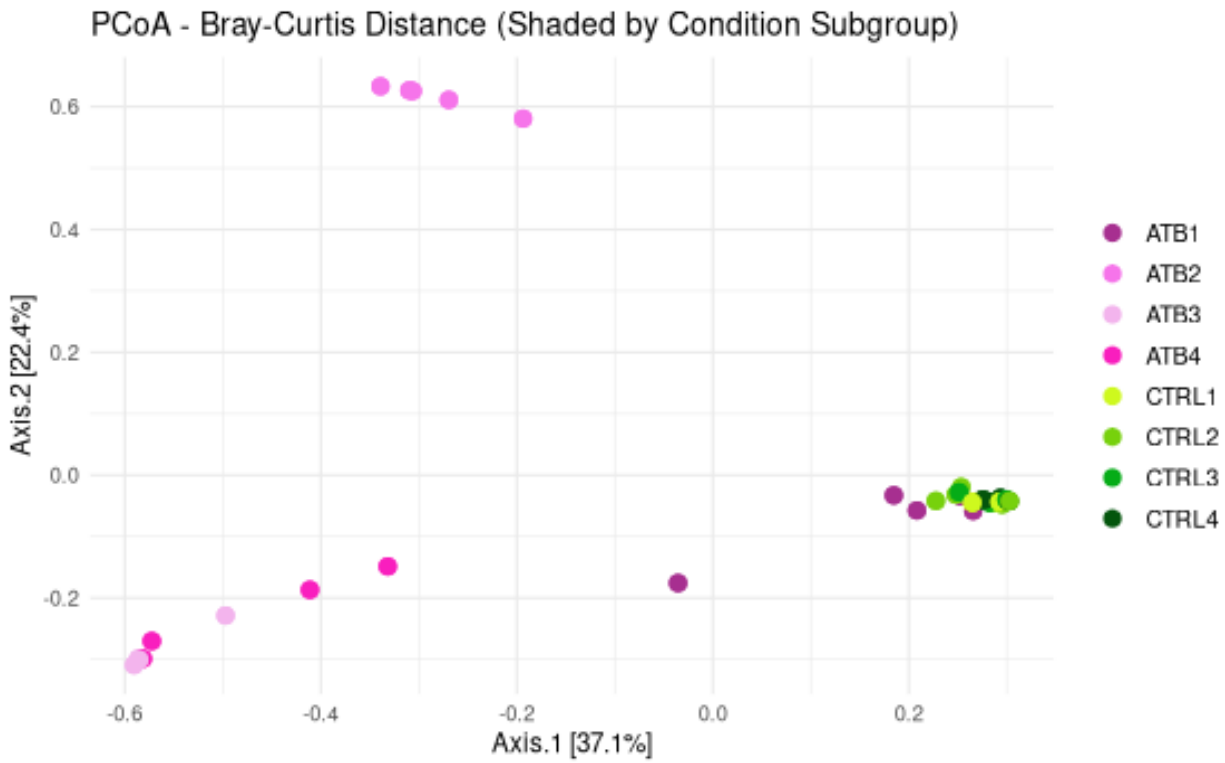

Figure 4. Beta diversity scatter plot, measures how similar or different the gut microbiome compositions are across our samples.

DISCUSSION

This analysis highlights the profound and dynamic effects of antibiotic treatment on the gut microbiome in a mouse model relevant to anorexia nervosa. Across all three-diversity metrics—Observed ASVs, Chao1, and Shannon index—mice exposed to antibiotics exhibited reduced microbial richness and evenness, particularly at midpoints (ATB2 and ATB3). Beta diversity analysis further revealed that antibiotic-treated mice developed distinct microbial communities, diverging significantly from the stable samples observed in control groups. These findings suggest that antibiotic exposure induces acute dysbiosis, but may allow for partial recovery over time, as evidenced by increased diversity metrics in ATB4.

The emergence of phyla such as *Pseudomonadota* and *Verrucomicrobiota* in antibiotic-treated groups underscores a compositional shift that may be linked to altered host-microbiota interactions. Furthermore, the ATB-treated mice were seen to have significantly reduced percentage abundance of Bacteroidota and Bacillota, which make

up much of the gut microbiome composition in the control groups. Lower levels of Bacteroidota have been linked with chronic gut inflammation – specifically with Inflammatory Bowel Disease [4].

With the gut microbiome's role in modulating neuropeptides related to appetite and behavior, these microbial shifts may have downstream implications for hypothalamic signaling and metabolic regulation.

LIMITATIONS

A limitation to our analysis was that the neuropeptide expression levels in the hypothalamus were assessed using quantitative PCR (qPCR) rather than transcriptome-wide RNA-sequencing (RNA-seq). Without raw sequencing data, we could not perform gene level quantification and investigate gene expression levels for the specific neuropeptides originally targeted in the paper, and our study was limited to studying microbiome composition differences across different experimental groups.

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