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Title

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1. Introduction

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the principal conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. Citing a journal paper [1]. Now citing a book reference [2,3] or other reference types [4–6]. Please use the command [7,8] for the following MDPI journals, which use author–date citation: Administrative Sciences, Arts, Econometrics, Economies, Genealogy, Humanities, IJFS, Journal of Intelligence, Journalism and Media, JRFM, Languages, Laws, Religions, Risks, Social Sciences, Literature.

2. Materials and Methods

2.1. Experimental Design

This study recruited Mexican women aged 18–45 years from gynecological consultations at ABC Medical Center. Participants were divided into two groups: cases and controls. Cases were women with recurrent vulvovaginal candidiasis (RVVC), defined as at least four episodes in the past 12 months, while controls were healthy women attending routine annual Pap smear consultations without a history of recurrent vaginitis.

Inclusion criteria for both groups required abstinence from sexual activity for 48 hours, no menstruation at the time of sample collection, no antibiotic use in the previous three months, and no diagnosis of diabetes mellitus or immunosuppressive disorders. Cases were additionally required to have a history of RVVC, while controls were limited to a history of one or fewer vaginitis episodes over their lifetime. Participants provided informed consent after receiving a detailed explanation of the study.

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Exclusion criteria included conditions contrary to the inclusion parameters, such as menstruation during sample collection, recent antibiotic use, or declining to participate. Controls were excluded if they had symptoms of vaginitis within the past six months or a history of more than one vaginitis episode diagnosed as vulvovaginal candidiasis. Cases were excluded if they did not meet the threshold for RVVC episodes or if they were outside the specified age range.

2.2. Vaginal Swab Sampling

Participants were positioned on a gynecological examination table with stirrups, and a vaginal speculum was inserted for sample collection. Cervicovaginal mucus was collected using Catch-All™ Sample Collection Swabs (Epicentre, Illumina) from the vaginal fornix. The vaginal pH was measured using Hydrion pH test strips (MicroEssential). The swab was then placed in a 2 mL tube containing sterile PBS solution, trimmed to fit, and stored at 4° C until DNA extraction.

2.3. DNA Extraction from Vaginal Swab Samples

DNA was extracted from vaginal swab samples using the GeneAll Exgene™ Stool SV Kit (GeneAll). Samples were first centrifuged for 5 minutes at 6000 rpm, and the supernatant was discarded. The pellet was resuspended in 1 mL of PBS buffer, vortexed for 1 minute, and incubated at room temperature for 30 seconds. After centrifugation at maximum speed for 2 minutes, the supernatant was discarded. The pellet was resuspended in 1.3 mL of Buffer FL, incubated for 5 minutes at room temperature, and centrifuged at $\geq$10,000 $\times g$ for 5 minutes. The supernatant was transferred to an EzPass™ filter column, centrifuged, and eluted into a clean 1.5 mL tube with 100 µL of Buffer EB. After further purification steps involving Buffer PB and Buffer NW, the final DNA elution was performed with 50 µL of Buffer EB. DNA integrity and concentration were verified using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and 0.5% agarose gel electrophoresis.

2.4. Amplification of 16S Ribosomal Gene (V3 and V4 Regions)

The presence of bacterial DNA in vaginal swab samples was confirmed by amplifying the V3 and V4 regions of the 16S ribosomal RNA gene using polymerase chain reaction (PCR). Reaction conditions and primers followed the protocols detailed in Tables 3 and 4, with a final reaction volume of 50 µL per sample.

Escherichia coli SK10019 was used as a positive control for bacterial DNA, and a reaction mix without template DNA served as the negative control. Amplifications were carried out using a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems). PCR products were resolved on 2% agarose gels stained with Midori Green Advanced dye, using TBE buffer. A molecular weight marker of 100 bp (Fermentas®) was included. Electrophoresis was conducted at 90 V for 50 minutes, and gels were imaged using a Molecular Imager® Gel Doc™ XR system (Bio-Rad).

2.5. Library Preparation for the V3 Region of the 16S rRNA Gene

Bacterial DNA presence was confirmed in vaginal samples, followed by library preparation targeting the V3 region of the 16S rRNA gene. A unique barcode sequence and sequencing adapters were incorporated using PCR under conditions detailed in Tables 5 and 6. Amplicon quality was verified via 2.0% agarose gel electrophoresis. Products of $\sim$281 bp were identified, and large-scale PCR was performed to increase product yield.

Equal concentrations of PCR products were pooled and further purified from preparative agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega). Final amplicon size ($\sim$281 bp) and DNA concentration were validated by analytical 2.0% agarose gel electrophoresis.

2.6. Amplification of the V5 Region of the 18S rRNA Gene

To detect yeast DNA, analytical PCR targeting the V5 region of the 18S rRNA gene was performed using high-fidelity Takara Ex Taq polymerase under conditions described in Tables 7 and 8. *Saccharomyces cerevisiae* S2886 served as a positive control, and a no-template reaction served as a negative control. Products were analyzed via 2.0% agarose gel electrophoresis.

2.7. Species Diagnosis of Candida via PCR Amplification

To determine the etiology of recurrent vulvovaginal candidiasis (RVVC) in case subjects and detect the presence of *Candida* in control subjects, PCR reactions were performed using species-specific primers for *C. albicans* and *C. glabrata*. These species were selected based on epidemiological prevalence, and primers for *Saccharomyces cerevisiae* were included due to its potential role in vulvovaginitis, which clinically resembles *Candida* infections.

Primers for *C. albicans* and *C. glabrata* targeted the Internal Transcribed Spacer (ITS) region, as described by Luo and Mitchell (2002). *S. cerevisiae* primers targeted the MEX67 gene, involved in mRNA nuclear export, following methods described by Muir et al. (2011). PCR reactions used high-fidelity Takara Ex Taq polymerase with conditions and primer sequences detailed in Tables 10–13. Positive controls included *C. albicans* ATCC, *C. glabrata* CBS138, and *S. cerevisiae* S2886, while negative controls used reaction mixtures without DNA templates.

Amplicons were resolved via 2.0% agarose gel electrophoresis stained with Midori Green Advanced dye, and products were visualized using the Molecular Imager® Gel Doc™ XR system (Bio-Rad). Amplicon sizes were confirmed against a 100 bp molecular weight marker.

PCR products were cloned using the GeneJET™ PCR Cloning Kit (Thermo Fisher), and plasmid DNA was extracted for capillary sequencing with pJET1 primers. Resulting sequences were analyzed using VECTOR NTI Advance™ and BLAST alignments to verify species-specific diagnostic accuracy. Figures 14–16 depict the pJET1.2blunt vector and the cloned sequences of each amplified product.

3. Results

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3.1. Distinct Microbial Profiles and Candida Prevalence in Vaginosis Cases vs. Controls

In this study, 38 controls and 57 cases met the inclusion criteria, of which 25 controls and 48 cases were successfully sequenced (Table A1). No significant differences were observed between groups regarding age, age at menarche, age of sexual life start, or vaginal pH, with other variables showing similar distributions between the groups (Figure A1). Notably, a subset of participants had used antibiotics (30.95% of cases and 43.86% of controls), and this factor was accounted for in subsequent analyses. Additionally, qPCR detection revealed significant differences in the prevalence of *Candida albicans* (90% in cases vs. 36.17% in controls) and *Candida glabrata* (0% in cases vs. 97.87% in controls) based on two-proportion z-tests (Table 1, Figure 1).

Table 1. Quantified variables in the studied subjects.

Variable	Control	Cases	*p*
Number	38	57	
Age	31.92 (±7.67) [38]	35.31 (±10.22) [51]	0.08
Menarche Age	12.53 (±1.59) [38]	12.64 (±1.44) [51]	0.71
Age Start Sexual Life	19.67 (±3.00) [36]	18.62 (±2.89) [48]	0.11
Contraceptive Method			
None	14/32 (43.75%)	29/57 (50.88%)	
Tubal ligation	1/32 (3.12%)	8/57 (14.03%)	
Condom	6/32 (18.75%)	10/57 (17.54%)	
Intrauterine device	2/32 (6.25%)	6/57 (10.53%)	
Oral	7/32 (21.88%)	4/57 (7.02%)	
Implant	2/32 (6.25%)	0/57 (0.00%)	
Menstrual Cycle Phase			
Luteal	19/32 (59.38%)	32/55 (58.18%)	
Follicular	11/32 (34.38%)	17/55 (30.91%)	
Ovulation	2/32 (6.25%)	6/55 (10.91%)	
Sex Partners			
1	15/32 (46.88%)	21/54 (38.89%)	
≥2	17/32 (53.13%)	33/54 (61.11%)	
Gestations			
0	17/31 (54.84%)	17/52 (32.69%)	
1-2	7/31 (22.58%)	18/52 (34.62%)	
≥3	7/31 (22.58%)	17/52 (32.69%)	
Delivers			
0	22/31 (70.97%)	32/52 (61.54%)	
1-2	4/31 (12.90%)	13/52 (25.00%)	
≥3	5/31 (16.13%)	7/52 (13.46%)	
Cesarean sections			
0	25/31 (80.65%)	30/52 (57.69%)	
1-2	5/31 (16.13%)	21/52 (40.38%)	
≥3	1/31 (3.23%)	1/52 (1.92%)	
Abortions			
0	29/31 (93.54%)	39/52 (75.00%)	
1-2	1/31 (3.22%)	12/52 (23.07%)	
≥3	1/3 (3.22%)	1/52 (1.92%)	
Vaginal pH			
mean pH	4.48 ± 0.57 [29]	4.78 ± 1.25 [43]	0.18
≤4	14/25 (56.00%)	17/40 (42.50%)	
≥5	11/25 (44.00%)	23/40 (57.50%)	
Smoking	8/42 (19.05%)	19/58 (33.33%)	
Antibiotics	13/42 (30.95%)	25/57 (43.86%)	
Antifungal	0/33 (0.00%)	5/57 (8.77%)	
Vulvovaginal Candidiasis episodes			
0	31/31 (100.00%)	0/52 (0.00%)	
1-2	0/31 (0.00%)	38/52 (73.08%)	
≥3	0/31 (0.00%)	14/52 (26.92%)	
qPCR *Candida* detection			
Candida albicans	27/30 (90.00%)	17/47 (36.17%)	9.93×10^{-6}
Candida glabrata	0/30 (0.00%)	46/47 (97.87%)	$<2.2 \times 10^{-16}$
Saccharomyces cerevisiae	27/30 (90.00%)	45/47 (95.74%)	0.6

Continuous data are represented as mean ± standard deviation, number individuals with available data is enclosed in square brackets. Categorical data are represented as proportions, percentages are enclosed in parentheses. For age, menarche age, start of sexual life and pH, student's *t*-test was applied. For qPCR detection results, a two-proportion *z*-test was used.

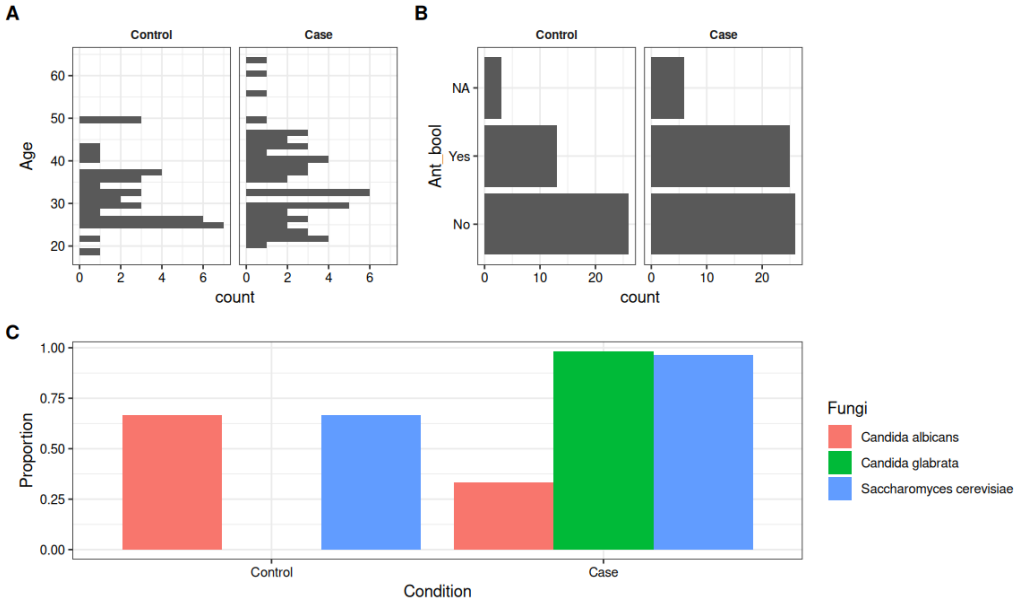

Figure 1. Main variables driving the study. **A.** Histogram depicting the age distribution between control and case groups. No statistical difference was found according to Student's t-test.**B.** Barplot showing antibiotic intake in women. **C.** Barplot showing proportion of control and case groups with three different Fungi detected by qPCR.

3.2. Antibiotic Intake and Patient Condition Have a Small Effect on Beta Diversity

Sequencing data were processed as outlined in the Methods section. For samples with low sequencing depth, re-sequencing was performed, and the resulting reads were combined by summation (Figure A2).

To investigate the impact of antibiotic use and patient condition on global bacterial diversity, both alpha and beta diversity metrics were analyzed. While alpha diversity appeared unaffected by these factors, beta diversity showed significant associations based on ADONIS tests. Weighted beta diversity was influenced primarily by patient condition (p = 0.042), whereas unweighted beta diversity was significantly associated with both patient condition (p = 0.012) and antibiotic use (p = 0.018). Despite these findings, the low R^2 values indicate that the observed effects are small (Figure 2).

Additionally, sequencing depth was examined in relation to antibiotic consumption to determine whether it contributed to a reduction in read counts; however, no evidence of such an effect was found (Figure A3).

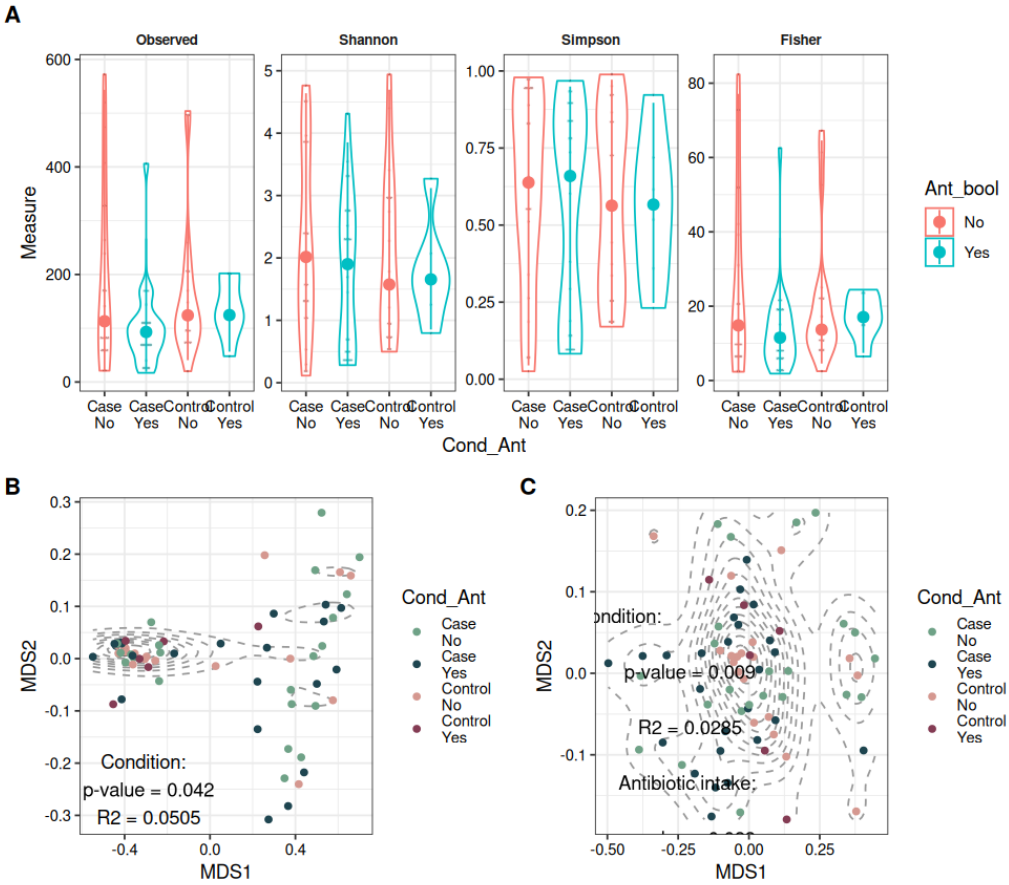

Figure 2. Diversity metrics for stratified groups, considering antibiotic intake and condition (case vs. control). **A.** Violin plot depicting alpha diversity metrics (Observed number of ASVs, Shannon, Simpson and Fisher) for the groups. **B.** Scatter plot showing Non-Metric Multidimensional Scaling (NMDS) for weighted Unifrac distance for beta diversity assesing in the groups. ADONIS was applied in order to account for the variance explained by Condition (p = 0.036, R^2 = 0.05) and Antibiotic intake (p = 0.39, R^2 = 0.01). **C.** Scatter plot showing Non-Metric Multidimensional Scaling (NMDS) for unweighted Unifrac distance for beta diversity assesing in the groups. ADONIS was applied in order to account for the variance explained by Condition (p = 0.012, R^2 = 0.03) and Antibiotic intake (p = 0.018, R^2 = 0.03).

3.3. Vaginal Microbiota Composition Reflects Patient Condition and Antibiotic Use

The composition of the vaginal microbiota was analyzed at both the phylum and genus levels to identify changes in key bacterial taxa across samples. Hierarchical clustering using weighted beta diversity was employed to compare taxa and assess similarities among bacterial communities in the groups.

Samples were first grouped by antibiotic intake and subsequently by patient condition. Significant alterations were observed in the *Firmicutes* ratio, particularly between cases and controls. Cases without antibiotic use exhibited the highest abundance of *Bacteroidota* and *Actinobacteriota*, whereas controls who had taken antibiotics were dominated by the *Firmicutes_D* phylum.

At the genus level, *Firmicutes_D* was predominantly represented by *Lactobacillus*, which also dominated controls with antibiotic use. In contrast, cases without antibiotic intake showed a higher prevalence of *Bifidobacterium*, while *Bacteroides_H* was found only in controls who had not taken antibiotics. Notably, *Fannyhessea* was characteristic of cases without antibiotic use, whereas *Sneathia* was specific to cases with antibiotic use.

Additionally, a marked increase in *Prevotella* was observed in cases where *Lactobacillus* was replaced by a diverse array of low-abundance taxa (<2%, grouped as "Other"). These

findings highlight the dynamic shifts in microbial composition associated with antibiotic use and patient condition (Figure 3).

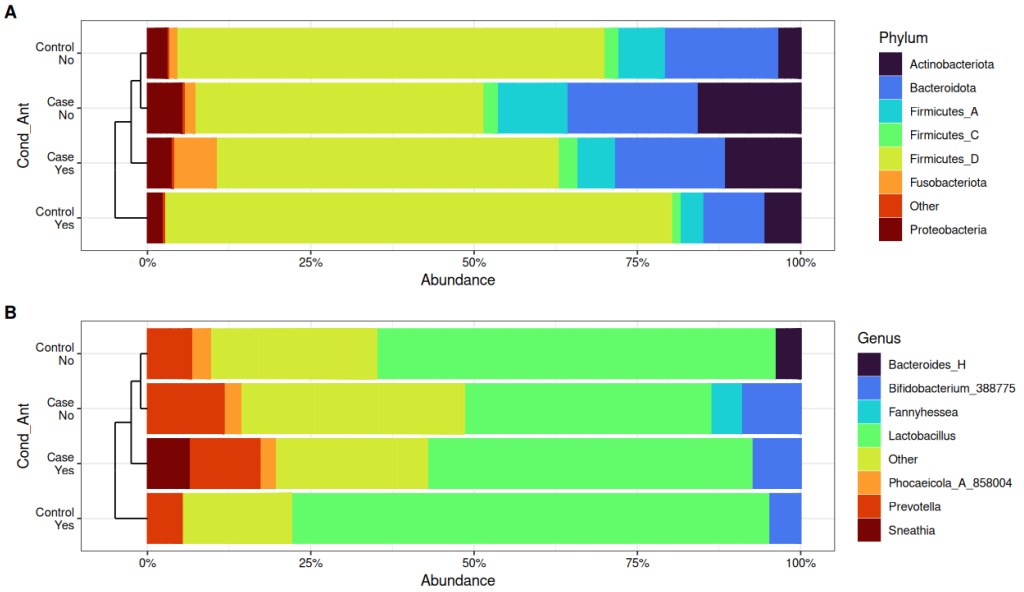

Figure 3. Relative abundance for stratified groups, considering antibiotic intake and condition (case vs. control). **A.** Phylum relative abundance as percentages. **B.** Genus relative abundance as percentages.

3.4. Lactobacillus and Other Key Bacterial Biomarkers Are Associated with a Healthy Vaginal Microbiota

To identify bacterial biomarkers associated with vaginosis, we performed differential abundance analyses while accounting for antibiotic intake. Two approaches were used: ALDEx2, with the formula $y \sim$ antibiotic intake + condition, and linear discriminant analysis (LEfSe), using condition as the class variable and antibiotic intake as the subclass.

The results from ALDEx2 are presented in Figure 4A, with corresponding square root-transformed relative abundance values shown in Figure 4B. In the control group, *Lactobacillus* and an unassigned taxon were prominent. Interestingly, two distinct ASVs classified as *Escherichia_710834* were identified in both the control and case groups.

LEfSe results are shown in Figure 4C, with the corresponding square root-transformed relative abundance values in Figure 4D. These findings corroborated the ALDEx2 results for *Lactobacillus*. Additionally, *Prevotella*, *Limosilactobacillus*, *Streptococcus*, and *Dialister* were predominant in controls, whereas two distinct *Escherichia_710834* ASVs, unassigned taxa, and *Cutibacterium* were characteristic of cases.

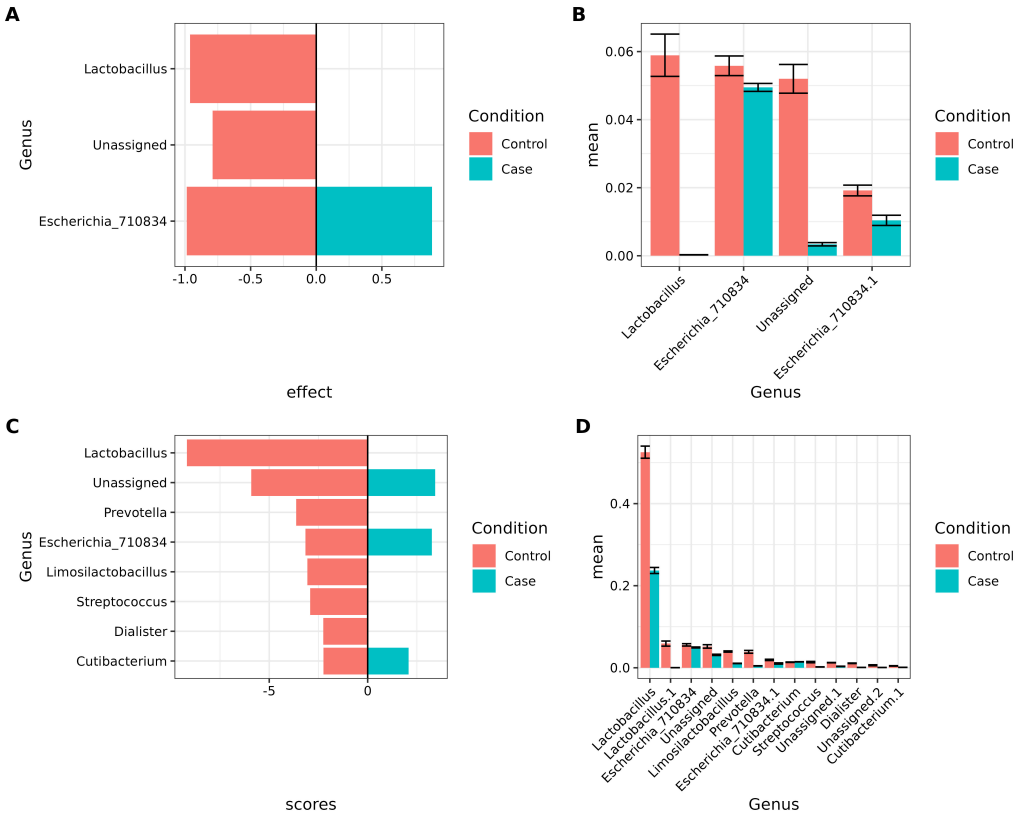

Figure 4. Important biomarker bacteria considering antibiotic intake and condition. A and B. Using ALDEx2 (y antibiotic intake + condition). C and D Using LEFSE (Class = condidion, Subclass = antibiotic intake). Only features with p-values which passed FDR correction were included in the graphs. **A.** Barplot showing ALDEx2 results for condition (case vs. control). X-axis shows the effect, Y-axis shows the genus. Negative values correspond to control, positive values correspond to cases. **B**. Barplot of mean sqrt of relative abundance of taxa found by ALDEx2. X-axis shows mean sqrt of relative abundance, Y-axis shows genus. **C.** Barplot showing LEFSE results for condition (case vs. control). X-axis shows the LDA scores, Y-axis shows the genus. Negative values correspond to control, positive values correspond to cases. **D**. Barplot of mean sqrt of relative abundance of taxa found by LEFSE. X-axis shows mean sqrt of relative abundance, Y-axis shows genus.

4. Discussion

Broad-spectrum antibiotics reduce gut microbiota diversity [**?**].

Clostridioides difficile (formerly known as Clostridium difficile) infection is an example of a disease brought about directly through antibiotic disruption of the gut microbiota /[**?**]

5. Conclusions

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6. Patents

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X.X.; writing—review and editing, X.X.; visualization, X.X.; supervision, X.X.; project administration, X.X.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.", please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
TLA	Three letter acronym
LD	Linear dichroism

Appendix A. Supplementary Tables

Table A1. Sequencing summary.

Parameter	Control (n = 27)	Case (n = 54)
Total reads	2,012,838	3,758,769
mean	74,549.56	69,606.83
sd	69,222.23	53,048.33
median	56,828.00	55,813.50
min	5,668	5,907
max	259,685	200,426

Appendix B. Supplementary Figures

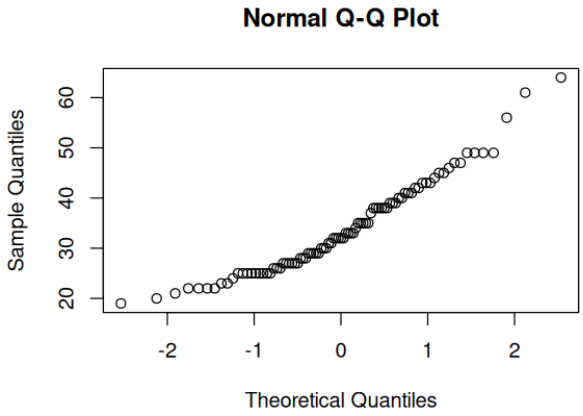

Figure A1. Normal Q-Q plot showing normal distribution of age in the studied women. Age was normally distributed according to Shapiro-Wilk test (p-value < 0.05).

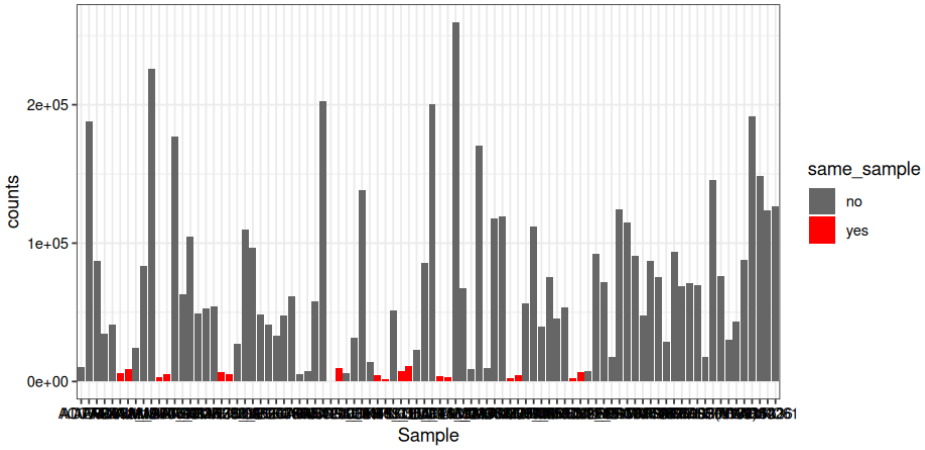

Figure A2. Barplot showing sequencing depth for each sample. Samples highlighted in red were sequenced twice and merged due they exhibited low sequencing depth.

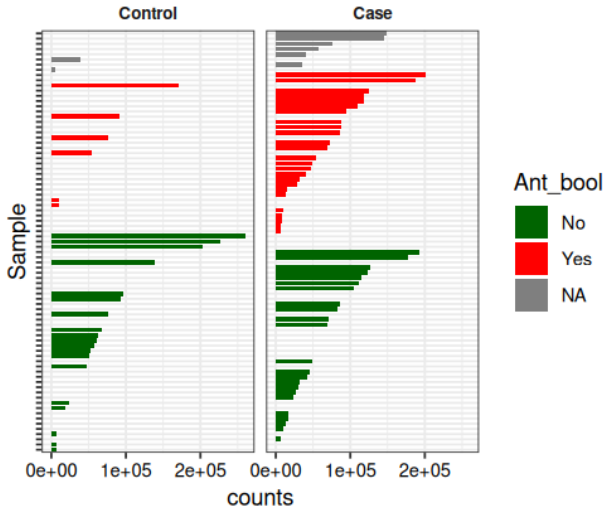

Figure A3. Barplot showing sample counts and antibiotic intake in both case and control groups.

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2. Author 2, L. The title of the cited contribution. In *The Book Title*; Editor 1, F., Editor 2, A., Eds.; Publishing House: City, Country, 2007; pp. 32–58.
3. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, 2008; pp. 154–196.
4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* year, *phrase indicating stage of publication (submitted; accepted; in press)*.
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