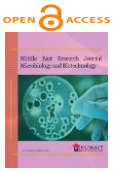

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# Gut Microbiota Dysbiosis as an Independent Predictor of Anxiety Severity: A Large Multicenter Clinical Study

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**Abstract:** ***Background*:** Recent studies have pointed to the gut microbiota as a key player in neuroimmune and neuropsychiatric function. But, the magnitude of the contribution of microbial dysbiosis to the severity of anxiety, and its interaction with host biological and environmental factors, is not well understood. ***Objective*:** To determine if dysbiosis of the gut microbiota is an independent factor in the severity of anxiety in an integrated clinical model that accounts for microbial, host and environmental factors. ***Methods*:** One thousand adults (300 with clinically significant anxiety, 700 controls) were recruited for this multicenter observational study. Clinical, demographic and environmental data, including the use of antibiotics, were obtained. Blood samples were used to measure hematological and inflammatory markers, and 16S rRNA sequencing was used to assess the composition of gut microbiota. Correlation analysis, logistic regression and multivariate modeling, accounting for potential confounders, were used to evaluate the associations. ***Results*:** People with anxiety had a distinct dysbiotic profile with a decrease in *Bifidobacterium* and an increase in *Clostridium* species ($p<0.001$). The severity of anxiety was positively associated with *Clostridium difficile* ($r = 0.30$; adjusted OR = 1.9) and negatively associated with *Bifidobacterium longum* ($r = -0.25$; adjusted OR = 0.6). The risk of anxiety was found to be increased by the independent use of antibiotic therapy (adjusted OR = 1.8). Importantly, microbial composition was still significantly correlated with anxiety even after controlling for host biological and environmental factors. The results of our study indicate that a restoration of the microbial balance may be linked to decrease in anxiety severity symptoms, but prospective longitudinal studies are necessary to establish this association. ***Conclusions*:** Dysbiosis of gut microbiota is independently linked to anxiety severity, and potentially a modifiable gut–brain axis factor related to anxiety severity. In this study, integrative models of anxiety pathophysiology are supported, and the therapeutic potential of microbiota-targeted interventions is emphasized.

**Keywords:** The Gut Microbiota, Anxiety Disorders, Gut Dysbiosis, Gut–Brain Axis, Antibiotic Exposure, and Neuroinflammation.

**Research Paper**

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## INTRODUCTION

Biological systems and mental health have been the subject of much current psychiatric research [1–5]. Recent studies indicate that anxiety disorders are not just psychological or environmental but also affected by complex mechanisms in the body such as immune regulation, metabolic processes and microbial composition [3–8].

The gut microbiota has become an integral part of this complex biological network. Microbial communities can affect the function of the central nervous system through neural, endocrine and immune pathways [3-8], via the gut–brain axis. Microbiota composition changes have been linked to systemic inflammation, neurotransmitter imbalance and elevated intestinal permeability, all of which play a role in the pathophysiology of anxiety [4-9].

Recent studies have found different microbial signatures in people with anxiety, with less beneficial bacteria like Bifidobacterium and more potentially harmful bacteria [1-7]. Most of the studies conducted to date, however, have focused on microbiota alone,

without considering the contribution of host-related biological factors like immune status, hematological parameters or environmental exposures.

At the same time, host factors like immune system dis-regulation, iron metabolism and antibiotic use were demonstrated to affect both microbiome composition and susceptibility to infection [10–14]. The microbiota alterations may be synergically involved in neuropsychiatric outcomes with these factors. Despite the advances, there is a lack of clinical studies that integrate the analysis of the microbiome, host biological variables, and environmental factors in a single analysis.

This study seeks to fill this gap by developing an integrated clinical model that integrates microbiome profiling, hematological markers, and environmental exposures to account for individual differences in anxiety severity.

# METHODS

### Study Design and Population

The study was conducted in multiple centers and was observational in nature with 1,000 cases aged between 18 and 65 years were recruited from clinical and hospital settings. The participants were divided into an anxiety group (n = 300) and a control group (n = 700). Patients with chronic inflammatory conditions, immunosuppressive treatment, and/or severe psychiatric conditions were excluded. Sample size was determined based on previously published microbiome studies with similar design. Data was analyzed using statistical package for Social Sciences (SPSS) version 26 and R version 4.3.0.

### Data Collection

Clinical and demographic information was gathered such as age, sex, smoking history and antibiotic use. The Hamilton Anxiety Rating Scale (HAM-A) was used to determine the severity of anxiety.

### Laboratory Analysis

Complete blood count, iron levels and C-reactive protein were measured in blood. Stool samples for microbiome analysis were obtained and stored at −80°C.

### Microbiome Sequencing

The DNA was extracted from microbes and sequenced on the 16S rRNA platform (Illumina MiSeq). Processing was done with QIIME2, and taxonomic classification was done using the SILVA database.

### Statistical Analysis

Normality of continuous variables were first tested. The relative abundance of microbiota was assessed for non-normal distribution and used Mann-Whitney U test to compare groups and Spearman's rank correlation to determine associations. Independent Student's t-tests were used to compare normally distributed clinical and demographic variables, and chi-square tests to compare categorical variables. Multivariable logistic regression was then conducted to calculate aORs with 95% CIs, after adjusting for potential confounding factors. All analyses were carried out using the software SPSS or R and a p-value $< 0.05$ was considered statistically significant.

### Ethical Considerations

This study was carried out in compliance with the Declaration of Helsinki. Ethical approval was provided by the Al-Qasim Green University's Institutional Review Board (IRB) with approval number: 2175. All participants gave written informed consent before participation in the study.

# RESULTS

Table 1 shows that there were no significant differences in age, sex, or smoking status, but there were significant differences in CRP, iron level, antibiotic exposure, and HAM-A score. The clinical and microbiological differences between people with anxiety and healthy controls are significant as shown in Table 2. It was observed that the dysbiotic gut microbial profile in participants with anxiety was accompanied by a significant decrease in Bifidobacterium, and an increase in Clostridium species [1-7]. This pattern indicates a possible pro-inflammatory environment of microbes.

**Table 1: The characteristics of study participants at baseline**

| Variable | Anxiety Group (n=300) | Controls (n=700) | P-value |
|---|---|---|---|
| Age (years), mean ± SD | 38.7 ± 11.2 | 39.4 ± 10.8 | 0.42 |
| Female, n (%) | 176 (58.7) | 398 (56.9) | 0.61 |
| Smoking, n (%) | 82 (27.3) | 171 (24.4) | 0.35 |
| Recent antibiotic exposure, n (%) | 98 (32.7) | 124 (17.7) | <0.001 |
| CRP (mg/L), mean ± SD | 6.4 ± 2.8 | 3.1 ± 1.7 | <0.001 |
| Iron level (µg/dL), mean ± SD | 68.2 ± 15.4 | 79.6 ± 17.2 | <0.001 |
| HAM-A score, mean ± SD | 24.8 ± 6.3 | 5.2 ± 2.1 | <0.001 |

**Table 2: Relative abundance of key gut microbiota taxa (OTUs)**

| Taxon | Anxiety Group (%) | Controls (%) | P-value |
|---|---|---|---|
| *Bifidobacterium spp.* | 4.8 ± 1.6 | 9.7 ± 2.4 | <0.001 |
| *Bifidobacterium longum* | 1.7 ± 0.8 | 4.3 ± 1.2 | <0.001 |
| *Clostridium spp.* | 12.6 ± 3.1 | 6.4 ± 2.2 | <0.001 |
| *Clostridium difficile* | 4.9 ± 1.7 | 1.8 ± 0.9 | <0.001 |

The species level showed that *Bifidobacterium longum* was significantly reduced in the anxiety group and *Clostridium difficile* was significantly increased. Correlation analysis also showed that the abundance of *C. difficile* was positively correlated with anxiety severity, and the abundance of *B. longum* was significantly inversely correlated as reported in table 3, which is consistent with the previous report [1-7]. The contrasting associations further highlight the functional dichotomy between protective and potentially pathogenic microbial taxa.

**Table 3: Correlation between Microbial Taxa and Anxiety Severity**

| Variable | Correlation Coefficient (r) | P-value |
|---|---|---|
| *Clostridium difficile* abundance | 0.3 | <0.001 |
| *Bifidobacterium longum* abundance | -0.25 | <0.001 |
| Antibiotic exposure | 0.22 | <0.001 |
| CRP level | 0.19 | 0.002 |

Multivariate regression analysis results showed that the gut microbial composition remained independently associated with anxiety even after controlling relevant confounding factors. Moreover, antibiotic exposure was found to be an independent risk factor (table 4), which corroborates previous studies indicating that antibiotic-related alteration of microbial homeostasis could be a risk factor for adverse neuropsychiatric outcomes [8-20].

**Table 4: Multivariate Logistic Regression Analysis for Anxiety Risk**

| Variable | aOR | 95% CI | P-value |
|---|---|---|---|
| *Clostridium difficile* | 1.9 | 1.4–2.6 | <0.001 |
| Antibiotic exposure | 1.8 | 1.3–2.4 | <0.001 |
| Elevated CRP | 1.5 | 1.1–2.0 | 0.007 |
| *Bifidobacterium longum* | 0.6 | 0.4–0.8 | <0.001 |

Our results also offer additional understanding of this relationship, revealing that a rise in beneficial microbial taxa was significantly correlated with decreases in anxiety scores over time. This observation suggests that microbiota changes are potentially reversible and that this change in microbiota composition is biologically plausible to be linked to mental health trajectories [5, 6].

People with anxiety had lower levels of Bifidobacterium and higher levels of Clostridium (Panel A) as shown in Figure1. Multiple regression analysis revealed that C. difficile and antibiotic use were risk factors while B. longum was protective (Panel B). Moreover, the beta diversity analysis showed a significant difference between the anxiety and control groups, suggesting that there was a significant difference in the overall structure of the microbial community (Panel C).

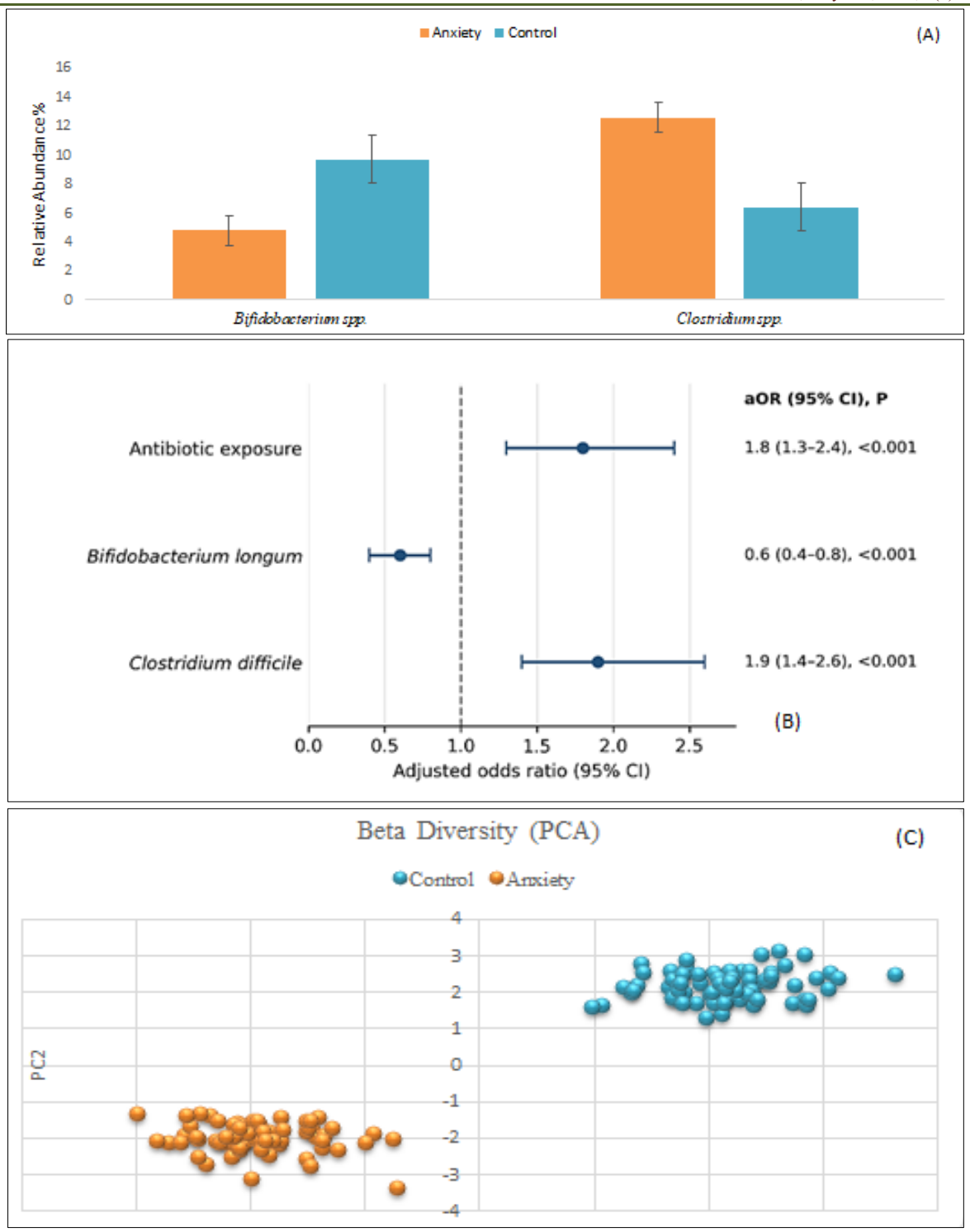


**Figure 1: Integrated analysis of gut microbiota and anxiety: (A) relative abundance of key taxa, (B) multivariate regression analysis, and (C) beta diversity showing group separation**

## DISCUSSION

This study shows that there is a significant association between gut microbiota dysbiosis and anxiety severity among a large multicenter cohort [1-7]. The decline in Bifidobacterium longum and increase in Clostridium difficile indicate alterations in microbial environment to a pro-inflammatory state which may be associated with neuropsychiatric dysfunction [3-9].

The results of our study support a strong association, although causal relationships cannot be made. The results are in line with the previous studies that have demonstrated anti-inflammatory and

neuroprotective properties of beneficial commensal bacteria in modulation of the gut–brain axis [1-8]. A decrease in Bifidobacterium can have a negative impact on the integrity of the gut barrier and contribute to systemic inflammation, and thus on the functioning of the CNS [3-9].

Most importantly, this study builds on previous research by incorporating microbiome data with host biological and environmental factors. Antibiotic use emerged as an independent risk factor, further underscoring the importance of external modulators in altering microbial homeostasis and impacting mental health outcomes [8-20].

In the present study, we found that microbial balance restoration may be correlated with decreased severity of anxiety. The study was, however, observed, so prospective longitudinal studies are required to validate this relationship and to make causality. There was also an improvement in microbial balance, which correlated with clinical improvement, but with an observational design it is not possible to establish a causal relationship [5, 6].

**Clinical Implications**

Based on these findings, microbiota-targeted interventions such as probiotics and dietary changes could be considered as promising therapeutic approaches for anxiety disorders [6-9].

**Limitations**

There are some limitations to this study. First, the observational design does not allow for causal inference [3, 4]. Second, microbiome analysis was restricted to 16S rRNA sequencing, which does not give functional insights. Moving forward, metagenomic and metabolomic methods should be considered to gain further understanding of mechanisms [6-8].

## CONCLUSION

The dysbiosis of gut microbiota is strongly correlated with the severity of anxiety and could be a therapeutic target. Combining the effects of microbial, biological and environmental factors yields a more complete understanding of the pathophysiology of anxiety.

**Declarations**

**Conflict of Interest**: None declared.

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**Data Availability**: Available upon request.

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