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ORIGINAL ARTICLE

# Evaluation of Gut Microbiota Variations and its Relationship with Interleukin-6 and β-Defensin-2 in Patients with Irritable Bowel Syndrome

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***Key words:***
***Functional gastrointestinal disorder, Microbial dysbiosis, Antimicrobial peptides, Cytokine profiling, Host-microbiota interaction***

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## ABSTRACT

***Background:*** *Irritable bowel syndrome (IBS) is a common gastrointestinal condition involving disruptions in gut microbial communities and immune system imbalances. Although previous research has examined microbial alterations in IBS, the connection between beneficial bacterial populations and immune indicators remains unclear.* ***Objectives:*** *This study aimed to explore changes in Bifidobacterium and Lactobacillus levels in IBS patients and their relationship with interleukin-6 (IL-6) and human β-defensin-2 (HBD-2).* ***Methodology:*** *A total of 55 individuals diagnosed with IBS (subcategorized into IBS-C, IBS-D, and IBS-M) and 26 healthy individuals participated in the study. Stool and blood samples were obtained. Quantitative real-time PCR was used to assess Bifidobacterium and Lactobacillus levels. ELISA was performed to measure serum IL-6 and fecal HBD-2 concentrations. Statistical analyses were conducted to compare groups and evaluate correlations between microbial and immune variables.* ***Results:*** *IBS patients exhibited a marked reduction in the levels of Bifidobacterium and Lactobacillus compared to healthy controls. Additionally, serum IL-6 and fecal HBD-2 concentrations were significantly elevated in the IBS group. A significant positive correlation was found between IL-6 and HBD-2 levels among IBS patients, while other correlations were not statistically significant. No meaningful differences in microbial or immune profiles were detected between IBS subtypes.* ***Conclusion:*** *The results suggest that gut microbial imbalance and immune activation, particularly increased IL-6 and HBD-2, may contribute to IBS pathogenesis. The observed link between IL-6 and HBD-2 supports a possible interaction between immune responses and antimicrobial peptide production, offering insight into potential microbiota-targeted therapies for IBS.*

## INTRODUCTION

Irritable bowel syndrome (IBS) is a widespread gastrointestinal disorder that presents with symptoms such as abdominal pain, bloating, and irregular bowel habits. It affects an estimated 10–20% of the global population. Although IBS was previously considered a purely functional disorder, emerging evidence has emphasized the role of gut microbiota alterations and immune system imbalances in its development[1].

Among the various microbial inhabitants of the gut, *Bifidobacterium* and *Lactobacillus* have garnered attention for their probiotic properties, which may influence gut health by modulating inflammation and enhancing gut barrier integrity[2].

Disruptions in gut microbiota, particularly reductions in beneficial bacteria like *Bifidobacterium* and *Lactobacillus*, have been linked to weakened intestinal barrier function, immune modulation, and altered gut-brain interactions[3]. Studies indicate that *Bifidobacterium* strains have a wide range of biological activities that promote health, such as protecting against infections, establishing a healthy microbiota in preterm infants, strengthening the intestinal gut barrier, and altering the host immunological response to create an anti-inflammatory milieu[4]. Furthermore, *Lactobacillus* regulates gut motility by producing neurotransmitters and interacting with the enteric nervous system[5].

Two crucial immune markers associated with IBS are IL-6 and HBD-2. IL-6 is a key cytokine in the gut, essential for maintaining intestinal homeostasis. It supports immune-epithelial communication, enhances immune responses by activating and recruiting immune cells during inflammation, and interacts with the nervous system, influencing enteric neurons and potentially affecting gastrointestinal motility, which is

especially significant for conditions like IBS[6]. HBD-2 is essential antimicrobial peptide that is critical for intestinal homeostasis and mucosal protection. It protects the intestinal epithelium from various pathogens while enhancing the integrity of epithelial tight junctions, which is vital for preventing infections. Additionally, HBD-2 helps maintain a balance between pathogenic and commensal bacteria, crucial for gut health. Its levels can increase during infections, indicating an active immune response, and it is suggested as a potential therapeutic tool for modulating immune responses in inflammatory diseases[7].

Recent studies highlight the significant role of gut microbiota in modulating immune function, with certain bacterial species demonstrating anti-inflammatory properties and offering protection against disturbances in intestinal immune regulation[8]. Gaining knowledge of the intricate relationships between the immune system and gut bacteria may help diagnose IBS more thoroughly and identify potential targets for therapeutic interventions[9].

The aim of this study is to assess the relationship between *Bifidobacterium*, *Lactobacillus*, IL-6, and HBD-2 in IBS patients compared to healthy controls. By investigating these interactions, we aim to expand current knowledge on IBS pathophysiology and identify potential targets for microbiota-based therapeutic approaches.

## METHODOLOGY

### Study Population

The study was conducted between March 2023 and April 2024 at Al Rajhi University Liver Hospital, Assiut University, and involved 55 individuals with IBS and 26 healthy participants. IBS was diagnosed using the Rome IV criteria, and patients were classified into three subtypes: constipation-predominant (IBS-C, n = 21), diarrhea-predominant (IBS-D, n = 27), and mixed-type IBS (IBS-M, n = 7). Subjects with recent antibiotic or probiotic use (within two months), a history of gastrointestinal surgery or comorbid gastrointestinal disorders (e.g., inflammatory bowel disease) were excluded.

### Ethical Approval

Approval for the study was obtained from the Human Ethics Committee, Faculty of Medicine, Assiut University (IRB number: 17101598), and informed written consent was secured from all participants prior to inclusion.

### Sample Collection

a. **Stool sample collection:** Fresh stool samples were collected in sterilized containers and stored immediately at –80°C.
b. **Blood sample collection and serum separation:** A 5 mL venous blood sample was drawn from each subject, centrifuged to separate the serum, which was then stored at -80°C for later cytokine assessment.

### Cytokine and Antimicrobial Peptide Measurement

- **Serum IL-6 Quantification**

IL-6 levels in serum samples were quantified using a sandwich ELISA kit (ELK Biotechnology Co., Ltd; Cat. No. ELK1156), according to the manufacturer's protocol. Optical density (OD) readings were obtained at 450 nm using a microplate reader.

- **Fecal HBD-2 Quantification**

Fecal HBD-2 levels were determined using a sandwich ELISA kit (SinoGeneClon Biotech Co., Ltd; Cat. No. SG-10208). Stool samples were homogenized in phosphate-buffered saline (PBS) and centrifuged to eliminate particulate matter. The resulting supernatants were analyzed using ELISA according to the kit instructions, with absorbance measured at 450 nm.

### DNA Extraction and Quantification of *Bifidobacterium* and *Lactobacillus* Gut Microbiota Using qPCR

Genomic DNA was isolated from 200 mg of each stool sample using the Easy Pure® Stool Genomic DNA Kit, following the manufacturer's guidelines. DNA yield and purity were assessed using a NanoDrop spectrophotometer (BioTek Epoch Microplate Spectrophotometer). Quantitative real-time PCR was conducted at the Medical Research Center, Assiut University, utilizing a TaqMan probe-based system on the 7500 Fast Real-Time PCR instrument. Specific primers and TaqMan probes targeting the 16S rDNA regions of *Lactobacillus* and *Bifidobacterium* were employed. The precise sequences of the primers and probes are detailed in Table 1[10].

**Table 1: 16S rDNA gene- targeted specific primers and TaqMan probes.**

| Targeted bacteria | Primer/ Prob | Oligonucleotide sequence (5'→3') | size (bp) | Product size (bp) |
|---|---|---|---|---|
| ***Lactobacillus* group** | primer F | GTCTGATGTGAAAGCCYTCG | 20 | **204** |
| | primer R | CCAGGGTATCTAATCCTGTTYG | 22 | |
| | Probe | YCACCGCTACACATGRAGTTCCACT | 25 | |
| ***Bifidobacterium* group** | **primer F** | **GGTTAACTCGGAGGAAGG** | **18** | **85** |
| | **primer R** | **GTACCGGCCATTGTAGCA** | **18** | |
| | **Probe** | **CGTCAGATCATCATGCCCCTTACG** | **24** | |

Each 20 µl qPCR reaction consisted of 0.5 µl forward primer, 0.5 µl reverse primer, 0.5 µl TaqMan probe, 12 µl of 2X ABT premix Master Mix, 1 µl of template DNA, and 5.5 µl of sterile ultra-pure water. The thermal cycling conditions included an initial hold at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds for each and annealing/extension at 60°C for 30 seconds. All reaction components aside from template DNA were present in negative controls. The reaction's specificity was validated by the fact that controls did not contain amplified DNA. The Ct values from standard curves were averaged to determine the absolute quantification, which was then expressed as the number of bacteria per gram of stool.

As positive controls, bacterial standard stains were obtained from Dr. Elhagag Ahmed Hassan of the Faculty of Science, Botany and Microbiology, Assiut University.

**Statistical Analysis**

Data analysis was done with SPSS version 20, with t-tests, ANOVA, and Pearson's correlation applied to assess differences and relationships between microbial and immune markers. Statistical significance was defined as a *p* value of < 0.05.

## RESULTS

The average age of IBS patients was 37.55 ± 7.51 years, compared to 36.62 ± 5.70 years in the control group, with no significant statistical difference (p = 0.489). Regarding gender, 41.8% of IBS patients were male and 58.2% female, while the control group had an equal proportion of males and females (50% each); this difference was also not statistically significant (p = 0.578) (Table 2).

The most prevalent IBS subtype in our study was IBS-D (diarrhea-predominant), comprising 49% of cases (n=27), followed by IBS-C (constipation-predominant) at 38% (n = 21), and IBS-M (mixed-type) at 13% (n = 7).

IL-6 concentrations were markedly higher in IBS patients than in the control group (22.75 ± 7.09 vs. 16.57 ± 3.73; p < 0.001). Similarly, levels of HBD-2 were significantly elevated in the IBS group (20.19 ± 3.69) compared to controls (16.85 ± 2.30; p < 0.001), as presented in Table 3.

Gut microbiota analysis by qPCR revealed a significant decrease in *Bifidobacterium* levels among IBS patients (11.19 ± 0.49 ng/µL) compared to the control group (11.87 ± 0.30 ng/µL; *p* < 0.001). Similarly, *Lactobacillus* levels were significantly lower in IBS patients (9.25 ± 2.63 ng/µL) than in controls (10.73 ± 1.08 ng/µL; *p* = 0.007), as presented in Table 4.

Analysis of different IBS subtypes revealed no statistically significant differences in IL-6 levels (p = 0.913), HBD-2 levels (p = 0.949), Bifidobacterium qPCR product (p = 0.185), or Lactobacillus qPCR product (p = 0.266), as presented in Table 5.

A strong positive correlation was observed between IL-6 and HBD-2 levels in the IBS group (r = 0.655, p < 0.001). Other correlations within the IBS group did not reach statistical significance (p > 0.05), as shown in Table 6. Likewise, no significant associations were found among the measured variables in the control group (Table 7).

**Table 2: Personal data of the studied groups**

| Personal data | IBS group (n= 55) | | Control group (n= 26) | | *P* -value |
|---|---|---|---|---|---|
| | No. | % | No. | % | |
| **Gender:** | | | | | |
| **Male** | 23 | 41.8% | 13 | 50.0% | 0.578 |
| **Female** | 32 | 58.2% | 13 | 50.0% | |
| **Age: (years)** | | | | | |
| **Mean ± SD** | 37.55 ± 7.51 | | 36.62 ± 5.70 | | 0.489 |
| **Range** | 22.0-53.0 | | 28.0-48.0 | | |

**Table 3: Serum IL-6 and Fecal HBD-2 concentration among the studied groups**

| The Variable | IBS group (n= 55) | Control group (n= 26) | *P* -value |
|---|---|---|---|
| **IL-6:** | | | |
| Mean ± SD | 22.75 ± 7.09 | 16.57 ± 3.73 | <0.001* |
| Range | 12.77-34.75 | 11.06-25.72 | |
| **HBD-2:** | | | |
| Mean ± SD | 20.19 ± 3.69 | 16.85 ± 2.30 | <0.001* |
| Range | 15.15-31.94 | 13.60-22.14 | |

**Table 4: Statistical comparison between IBS and control group as regard *Bifidobacterium* and *Lactobacillus* qPCR product.**

| The Variable | IBS group (n= 55) | Control group (n= 26) | *p* -value |
|---|---|---|---|
| ***Bifidobacterium*:** | | | |
| **Mean ± SD** | 11.19 ± 0.49 | 11.87 ± 0.30 | <0.001* |
| **Range** | 10.06-11.92 | 10.68-12.33 | |
| ***Lactobacillus*:** | | | |
| **Mean ± SD** | 9.25 ± 2.63 | 10.73 ± 1.08 | 0.007* |
| **Range** | 5.51-12.75 | 8.37-12.62 | |

**Table 5: IL-6 and HBD-2 concentration and qPCR product based on type of IBS**

| The variable | Types of IBS | | | *P* -value |
|---|---|---|---|---|
| | Constipation (n= 21) | Diarrhea (n= 27) | Mixed (n= 7) | |
| **IL-6:** | | | | |
| Mean ± SD | 22.44 ± 7.44 | 23.15 ± 6.49 | 22.09 ± 9.11 | 0.913 |
| Range | 13.45-34.75 | 13.64-34.69 | 12.77-31.39 | |
| **HBD-2:** | | | | |
| Mean ± SD | 20.26 ± 4.15 | 20.05 ± 3.34 | 20.54 ± 4.07 | 0.949 |
| Range | 15.15-31.94 | 15.81-27.48 | 16.60-26.34 | |
| ***Bifidobacterium*:** | | | | |
| **Mean ± SD** | 11.27 ± 0.49 | 11.08 ± 0.50 | 11.40 ± 0.36 | 0.185 |
| **Range** | 10.07-11.92 | 10.06-11.85 | 10.99-11.77 | |
| ***Lactobacillus*:** | | | | |
| **Mean ± SD** | 8.51 ± 2.83 | 9.74 ± 2.39 | 9.56 ± 2.83 | 0.266 |
| **Range** | 5.51-12.75 | 5.51-12.30 | 5.51-12.25 | |

**Table 6: Correlation between IL-6, HBD-2, *Bifidobacterium and Lactobacillus* among IBS group**

| The variable | | IL-6 | HBD-2 | *Bifidobacterium* | *Lactobacillus* |
|---|---|---|---|---|---|
| **IL-6** | **r-value** | | | | |
| | ***p* -value** | | | | |
| **HBD-2** | **r-value** | 0.655 | | | |
| | ***p* -value** | <0.001* | | | |
| ***Bifidobacterium*** | **r-value** | -0.043 | 0.033 | | |
| | ***p* -value** | 0.757 | 0.812 | | |
| ***Lactobacillus*** | **r-value** | -0.042 | -0.126 | -0.081 | |
| | ***p* -value** | 0.761 | 0.361 | 0.558 | |

**Table 7: Correlation between IL-6, HBD-2, *Bifidobacterium and Lactobacillus* among control group**

| The variable | | IL-6 | HBD-2 | *Bifidobacterium* | *Lactobacillus* |
|---|---|---|---|---|---|
| **IL-6** | **r-value** | | | | |
| | ***p* -value** | | | | |
| **HBD-2** | **r-value** | -0.329 | | | |
| | ***p* -value** | 0.100 | | | |
| ***Bifidobacterium*** | **r-value** | 0.244 | 0.147 | | |
| | ***p* -value** | 0.230 | 0.474 | | |
| ***Lactobacillus*** | **r-value** | -0.037 | -0.039 | -0.019 | |
| | ***p* -value** | 0.858 | 0.851 | 0.927 | |

**P* value is significant < 0.05.
***Correlation is significant < 0.05.

## DISCUSSION

Irritable bowel syndrome (IBS) is a prevalent functional gastrointestinal condition that affects millions worldwide, marked by persistent abdominal discomfort, bloating, and changes in bowel habits. Despite its substantial global impact, the underlying pathophysiology of IBS remains poorly understood. However, increasing evidence suggests that complex interactions among the gut microbiota, immune response, and intestinal barrier integrity play a key role in its development[11].

The immune system appears to play a pivotal role in the pathophysiology of IBS, particularly through the modulation of inflammatory cytokines. In this study, as shown in Table 3, IL-6 levels were significantly elevated in IBS patients compared to healthy controls, indicating immune activation and the presence of low-grade inflammation. These findings are in line with previous research, including studies by Ismail et al.[12], Vara et al.[13], and Linsalata et al.[14], which also reported increased IL-6 levels in IBS patients. Such elevations have often been associated with gut barrier dysfunction and microbial influences, including infections such as *Blastocystis*. However, other studies, like those by Mujagic et al.[15] and Mitselou et al.[6], observed either decreased IL-6 levels or localized increases, indicating that IL-6 involvement may vary depending on IBS subtype, gut barrier integrity, and microbial composition. These discrepancies highlight the heterogeneous nature of IBS and suggest that both immune activation and dysregulation may coexist, emphasizing the complex interplay between the immune system, gut microbiota, and epithelial barrier function in IBS.

Similarly, our study demonstrated elevated HBD-2 levels in IBS patients, indicating enhanced activation of the innate immune response and its potential involvement in low-grade inflammation. Supporting our results, Langhorst et al.[16] reported increased fecal HBD-2 levels in IBS patients, comparable to those observed in active ulcerative colitis, suggesting mucosal immune activation even in the absence of overt inflammation. In contrast, Kermani et al.[17] found reduced HBD-2 expression in the sigmoid mucosa of IBS patients, implying that HBD-2 may be downregulated in inflamed tissues or expressed primarily in non-inflamed regions. These contrasting findings underscore the complex and context-dependent role of HBD-2 in IBS pathogenesis and highlight the need for further research to clarify its regulatory mechanisms and clinical significance.

Our study revealed a significant reduction in *Bifidobacterium* (11.19 ± 0.49 ng/µL vs. 11.87 ± 0.30 ng/µL; $p < 0.001$) and *Lactobacillus* (9.25 ± 2.63 vs. 10.73 ± 1.08; $p = 0.007$) levels in IBS patients compared to healthy controls, suggesting a clear presence of gut dysbiosis in IBS. These findings align with prior studies by Ji et al.[18], Shukla et al.[19], Transeth et al.[20], Naseri et al.[21], and the systematic review by Zhuang et al.[22], all of which reported reduced abundance of these beneficial genera in IBS, suggesting their critical role in maintaining gut barrier function, immune modulation, and microbial balance. Specifically, the decline in *Bifidobacterium* may impair epithelial integrity and promote inflammation, contributing to IBS symptomatology. Conversely, Maccaferri et al.[23] found an increased abundance, and Mättö et al.[24] observed no significant difference, underscoring the variability in microbial findings across populations and methodologies. These contrasting results highlight the complexity and heterogeneity of gut microbiota alterations in IBS and support the growing recognition of microbiota-targeted strategies in its management.

Among IBS subtypes, our results align with those of Zhang et al.[25], who found no significant differences in gut microbiota composition. As presented in Table 3, there were no significant variations in *Bifidobacterium* ($p = 0.185$) or *Lactobacillus* ($p = 0.266$) levels across IBS-C, IBS-D, and IBS-M subtypes. Furthermore, Zhang et al.[25] reported no significant differences in inflammatory factor levels between different IBS phenotypes ($p > 0.05$). Similarly, our study demonstrated that different IBS subtypes exhibited no significant differences in IL-6 levels ($p = 0.913$) or HBD-2 levels ($p = 0.949$). These findings suggest that other factors, such as immune responses or gut-brain interactions, might play a more prominent role in the differences observed between IBS subtypes.

The correlation analysis revealed a significant positive correlation between IL-6 and HBD-2 levels in IBS patients ($r = 0.655$, $p < 0.001$). However, no significant correlations were found within the IBS group or in the control group.

This significant positive correlation, suggesting a dynamic interplay between immune activation and mucosal defense mechanisms. Elevated HBD-2 levels, known to be associated with inflammatory conditions, and its ability to induce cytokine production, including IL-6 in peripheral blood mononuclear cells (PBMCs). Boniotto et al, point to a potential positive feedback loop that may exacerbate inflammation in IBS. Furthermore, HBD-2 expression is upregulated in response to IL-6 and other pro-inflammatory cytokines, reinforcing this interaction. This positive correlation highlights the inflammatory underpinnings of IBS and suggests that targeting IL-6 and HBD-2 could open new therapeutic avenues, as IL-6 is already known to promote inflammation in other gastrointestinal diseases such as ulcerative colitis and Crohn's disease via mechanisms like T-cell survival through the STAT3 pathway[26,27].

The lack of significant correlations between IL-6 and HBD-2 in the control group likely reflects their distinct immune roles. IL-6 regulates systemic inflammation and immune balance, while HBD-2 is locally induced by specific bacterial and fungal pathogens. In healthy individuals, without active infection or inflammation, these pathways function independently, explaining the absence of a correlation between IL-6 and HBD-2[28-30].

## CONCLUSION

Our study demonstrated that IBS patients exhibit significantly higher levels of IL-6 and HBD-2 alongside a notable reduction in *Bifidobacterium* and *Lactobacillus* compared to healthy controls, suggesting the involvement of low-grade inflammation and gut dysbiosis in IBS pathogenesis. A positive correlation between IL-6 and HBD-2 levels further supports the interaction between immune activation and mucosal defense mechanisms. No significant differences were detected among different IBS subtypes regarding microbial or inflammatory markers, indicating that immune and microbial disturbances may be common features across IBS types. These findings reinforce the concept that IBS involves both immune dysregulation and microbiota alterations, highlighting the potential of targeting gut microbiota and inflammatory pathways for future therapeutic interventions.

**Declarations:**

The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.

Each participant signed an informed consent before participating in the study.

This article has not been published anywhere and is not currently under consideration by another journal or publisher.

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