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ORIGINAL RESEARCH

Exploring connection between Gut microflora and inflammatory bowel disease

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ABSTRACT

Introduction: Inflammatory bowel disease (IBD) is a complex condition with an unknown etiology, although dysbiosis of the gut microbiota has been proposed as a potential contributing factor. Despite this, the specific changes in gut microbiota in IBD patients, particularly during active flare-ups and remission , compared to healthy individuals, are not yet well understood. The goal of this study was to explore the microbial alterations occurring in the gut during both the exacerbation and remission phases of IBD and to contrast these changes with those in healthy control subjects. **Patients and Methods:** The current study involved eleven patients with inflammatory bowel disease (IBD) and nine healthy control subjects. Nine IBD patients were available for follow-up, enabling repeat sample collection after confirming their disease status. This was an observational-analytical study, aimed to examine changes in the gut microbiota of IBD patients without any interventions. **Observation & Results**: Our findings show that the total bacterial count was significantly higher in the non-IBD control group compared to both the exacerbated IBD group and the follow-up group. In exacerbated IBD patients, bacterial counts were lower than during remission, with qPCR analysis revealing a sharp decline in bacterial count during flare-ups, followed by an increase as patients transitioned into remission. **Conclusion**: Despite the ongoing uncertainty about the link between gut microflora and inflammatory bowel disease (IBD), our study and previous reports suggest that dysbiosis in gut microbiota, may contribute to acute IBD exacerbations. A reduction in microbiota , capable of producing short-chain fatty acids (SCFAs) could promote intestinal inflammation, while an increase in harmful bacterial communities may worsen the disease. Future research on restoring beneficial bacteria with prebiotics and probiotics may offer insights into improving IBD prognosis.

Key words: Inflammatory Bowel Disease (IBD), Dysbiosis, Exacerbation, Remission, Short-Chain Fatty Acids (SCFAs), Bacterial Count.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, immune-mediated gastrointestinal disorder that causes significant discomfort and often requires surgical intervention for many patients. It primarily affects adults in their prime working years, typically between the ages of 15-45, making it an economically significant disease (Fiocchi, 2015; Wills et al., 2014; Xavier & Podolsky, 2007). IBD encompasses two major conditions: ulcerative colitis (UC) and Crohn's disease (CD). UC is limited to the colon, whereas CD can affect any part of the gastrointestinal tract, although it most commonly involves the small intestine or colon. The disease manifests in two distinct phases: exacerbation (active phase), characterized by intense inflammation and symptom flare-ups, and remission (inactive phase), where inflammation subsides and symptoms lessen (Xavier & Podolsky, 2007; Wills et al., 2014; Wallace et al., 2011; Tong et al., 2013; Sepehri et al., 2007).IBD is believed to result from a combination of three primary factors: 1) a genetically susceptible host, 2) immune system dysfunction, and 3) microbial dysbiosis (Sepehri et al., 2007; Robles-Alonso & Guarner, 2014; Rehman et al., 2010; Paul, Verma, & Verma, 2007; Organ & An, 2004). Recent research underscores the pivotal role of the intestinal microbiota in the pathogenesis of IBD. In genetically predisposed individuals, an altered microbiota triggers an inappropriate immune response that leads to chronic intestinal inflammation. This has led to a growing interest in monitoring microbial changes in IBD patients as a means of understanding the disease and developing potential therapeutic interventions (DuPont & DuPont, 2011; Faith et al., 2013).Historically, IBD has had the highest incidence rates in developed countries, particularly in North America and Europe. However, recent studies suggest

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a rising incidence of IBD, particularly UC, in developing regions such as Japan, South Korea, Singapore, northern India, and parts of Latin America—areas once thought to have low rates of the disease (Chichlowski & Hale, 2008; DuPont & DuPont, 2011; Walker et al., 2011; Wallace et al., 2011). This trend indicates that IBD may become a significant health issue for gastroenterologists in Asia in the coming decades (Anders Ekbom, 2011).The pathogenesis of IBD is thought to involve complex interactions between exogenous factors, such as the composition of the gut microbiota, and endogenous host factors, including the function of the intestinal epithelial barrier and the immune system (Cho, 2008; Lyra et al., 2012; McFall-Ngai et al., 2013; Goulet, 2015). In healthy individuals, the immune system maintains a balanced relationship with the gut microbiota, allowing immune tolerance to commensal bacteria and dietary antigens. In IBD, however, this balance is disrupted, either due to a breakdown in immune regulation or dysbiosis of the microbiota, leading to chronic intestinal inflammation (Cho, 2008; Lyra et al., 2012; Cader & Kaser, 2013; McFall-Ngai et al., 2013).At birth, the human gut is sterile, and microorganisms begin to colonize the gut immediately after delivery. This process continues throughout life, with the gastrointestinal (GI) tract harboring a diverse and dense community of microbes—particularly in the colon. The gut microbiota plays crucial roles in immune system development, metabolism, and protection against pathogens. Disruptions in this microbial community, such as those caused by poor diet, infections, or environmental factors, can provoke immune system activation, leading to inflammatory diseases like IBD (Wallace et al., 2011; Vanhoutte et al., 2004; Ramakrishna, 2013).The human gut contains up to 100 trillion microbial cells, with the highest diversity found in the colon, where microbial densities can reach 10^{11}–10^{12} cells per milliliter (Faith et al., 2013; Bäckhed et al., 2005). The dominant bacterial phyla in the gut microbiota are Firmicutes and Bacteroidetes, with smaller amounts of Proteobacteria. In IBD, the mucosa-associated microbiota, which is in close contact with the gut epithelium, appears to play a key role in disease development. In healthy individuals, the mucosa-associated microbiota is relatively sterile, but in IBD patients, particularly those with Crohn's disease, there is a marked increase in bacterial colonization (Bäckhed et al., 2005; Faith et al., 2013).This dysbiosis—the imbalance in the gut microbiota—is thought to exacerbate inflammation in IBD, underscoring the relevance of studying the mucosal microbiota in understanding the disease process. Further research into the gut microbiota and its interactions with the host immune system is crucial for developing new diagnostic and therapeutic strategies for IBD.

MATERIALS & METHODS

Sample Acquisition

The samples for this study were collected from adult male and female patients diagnosed with inflammatory bowel disease over a period of twelve months (between September 2024 to September 2024). Patients were recruited from Shadan Hospital, Hyderabad, Telangana , India.Patients who presented with symptoms suggestive of inflammatory bowel disease (IBD) and were confirmed to have the condition by a gastroenterologist were included in the study. Participation was entirely voluntary, and all individuals were given an equal opportunity to enroll. Neither the researchers nor the medical staff influenced or pressured any patient or control participant to take part in the study.Individuals who tested positive for inflammatory bowel disease (IBD) based on histological and radiological findings were selected for endoscopic evaluation of the gastrointestinal tract. Following the endoscopy, the gastroenterologist provided biopsy samples, and stool and blood samples were simultaneously collected from the same patient. Patient selection was based on criteria established by the American Gastroenterological Association (AGA) and the Crohn's and Colitis Foundation of India (CCFI). The patients were classified into two groups: those in the exacerbated phase of IBD and those in the remission phase, the latter of whom were considered follow-up patients after receiving treatment.

The clinical criteria for diagnosing IBD were based on the guidelines issued by the American Gastroenterological Association (AGA) and the Crohn's and Colitis Foundation of America (CCFA). Additionally, the following criteria were applied:

- Age range: 20 to 50 years
- Both male and female participants
- No history of systemic illnesses such as diabetes or cardiovascular diseases
- No other gastrointestinal complications

Over the course of 6 months, fourteen patients were diagnosed with IBD. Biopsy, stool, and blood samples were successfully collected from eleven of these patients, while the remaining three did not consent to participate. During the follow-up phase, when the disease was in remission, samples were obtained from nine patients, as two patients did not return for the follow-up study.

For the non-IBD control group, it was decided that samples would be collected from eight or nine individuals. In the current study, data from nine non-IBD control participants were included.The study involved a comprehensive approach to sample collection, which included:

- Biopsy Samples: Mucosal biopsy samples were collected from the same mucosal area, each approximately 1 × 2 mm in size. Each biopsy was collected in 1 ml of sterile phosphate buffer saline solution. The samples were weighed and processed for DNA extraction almost immediately after

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collection. The extracted DNA was preserved at -20 °C until further experiments were conducted .

- Stool Samples: Alongside biopsy samples, stool samples were collected from the patients. This was done to analyze the gut microbiota composition and its potential association with IBD .
- Blood Samples: Blood samples were also collected from the patients to assess any systemic changes associated with IBD and to complement the findings from the biopsy and stool analyses .

Cases were selected based on the following inclusion and exclusion criteria:

Inclusion Criteria

- Abdominal cramps or pain
- Chronic diarrhea with blood in the stool
- Loss of appetite

Exclusion Criteria

- Individuals who had received antibiotic treatment within the last 90 days
- Individuals with other intestinal diseases, such as celiac disease

Risk Factors: including adverse event monitoring: NIL

To calculate the sample size for the study, the prevalence of inflammatory bowel disease (IBD) would typically need to be estimated. However, exact prevalence data for IBD were unavailable , assistance of a statistician, that all patients diagnosed with IBD within the first 2 months of the study would be invited to participate, provided they gave their consent. Follow-up samples were to be collected later in the study .In the current study, all techniques, reagents, and instruments were used in accordance with international standards and the manufacturer's instructions. To minimize errors and biases, including sampling errors and inter-observer variability, we adhered to the guidelines provided by the Crohn's and Colitis Foundation of America (CCFA). Additionally, clinical diagnoses and sample collection were exclusively carried out by a dedicated gastroenterologist to ensure consistency and accuracy.

Study Design

This study was designed as an observational study, as no interventions were implemented by the investigators. The goal was to observe and characterize the changes in the gut microbiota without altering the natural progression of the disease. Observational studies are generally classified as either descriptive or analytical. Given that our study aimed to analyze the variations in the gut microbiota and examine its association with the exacerbation of inflammatory bowel disease, it was categorized as an observational-analytical study, in line with the guidelines set by the WHO (Fathalla & Fathalla, 2004).

Quantitative Real-Time PCR for Total Bacterial Count

Total bacterial quantification in biopsies and stool samples was performed using quantitative real-time PCR (qPCR). The qPCR reactions were conducted using the Applied Biosystems 7300 Real-Time PCR System (Foster City, CA) along with the Sequence Detection System (SDS) Software (version 1.4). Each PCR reaction was carried out in a final volume of 20 µl, containing 1× SYBR Green qPCR Master Mix (Qiagen), 0.5 µM of each primer, and 40 ng of purified genomic DNA from the colonic mucosa. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing at 60°C for 30 seconds, and DNA extension at 72°C for 90 seconds. A final melt curve analysis was performed to confirm the specificity of the amplification products.For each sample, triplicate reactions were performed, and appropriate standards were included in each run. The bacterial 16S rRNA gene was amplified using the forward primer 5′-TCCTACGGGAGGCAGCAGT-3′ and the reverse primer 5′-GGACTACCAGGGTATCTAATCCTGTT-3′ (Nadkarni et al., 2002). A serial tenfold dilution of extracted DNA from a pure *Bacteroides fragilis* (CCUG 4856) culture was used to generate a standard curve. The quantification of bacterial abundance was based on a comparison of the fluorescence threshold with the standard curve. The relative bacterial abundance in UC patients was calculated as a 'fold change' in comparison to control groups (Walujkar et al., 2014).All qPCR plates included a 'no template' negative control to check for contamination or primer-dimer artifacts. Additionally, melt curve analysis was performed after each assay to ensure that the fluorescence signal originated exclusively from the specific PCR product and not from non-specific amplification.

RESULTS

The current study comprised a total of twenty subjects, divided into two groups . Eleven Patients with Inflammatory Bowel Disease (IBD) and nine control subjects . The control group consisted of individuals who did not have any history of IBD or related gastrointestinal diseases. These subjects were classified as "normal" based on endoscopic, radiologic, and pathologic evaluations, ensuring that they had no evidence of disease in the small or large bowel.The commonest clinical presentations in the patients with IBD were abnormal bowel frequencies, blood in stool, loss of appetite, weight loss, abdominal cramps, headache and fever, anxiety & depression . In the control group patients , it was observed that some of the individuals had shown positive symptoms for blood in stool, uneven frequencies of diarrhea, and loss of appetite, but none of these non-IBD control group individuals were found to be suffering from any gastrointestinal tract infections.

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Microbial count quantification

The total bacterial count in mucosal biopsy samples from three different groups was measured using quantitative PCR (qPCR). In the control group, the bacterial count was 0.9×10^{11}/ml. In contrast, patients experiencing acute exacerbations had a significantly lower bacterial count, with a value of 3.12×10^{6}/ml . During the follow-up sampling, which corresponded to the remission stage, the bacterial count slightly increased to 2.96×10^{7}/ml.

DISCUSSION

Inflammatory bowel disease (IBD) has emerged as a significant healthcare issue in the modern world, with the highest incidence rates reported in developed countries. Recently, however, there has been a notable increase in cases in developing countries as well.(Eppinga, Fuhler, Peppelenbosch, & Hecht, 2016; Ray, 2016; Cosnes, Gower-Rousseau, Seksik, & Cortot, 2011; Kirsner, 1995; McFall-Ngai et al., 2013) IBD primarily manifests in two forms: Crohn's disease (CD) and ulcerative colitis (UC), both of which are chronic, recurrent inflammatory conditions. In Crohn's disease, inflammation affects the entire thickness of the bowel wall (transmural) and can involve any part of the gastrointestinal tract. CD is often associated with complications such as fistulas, strictures, and abscesses. In contrast, ulcerative colitis is characterized by mucosal inflammation, typically confined to the colon.(Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; Frank et al., 2007; Ray, 2016; Shetty, Marathe, & Shouche, 2013). The exact cause of IBD remains largely unknown. However, recent studies suggest that its pathogenesis is influenced by a combination of environmental factors, disruptions in the intestinal microbiota, abnormal immune responses, and genetic susceptibility.(Conte et al., 2006; Cox, Cookson, & Moffatt, 2013a; Disease, 2003; Kirsner, 1995; Marteau, Seksik, & Shanahan, 2003). Both earlier and recent studies on IBD, including research by Melissa Friswell et al. from the UK and Julien Matricon et al. from France, highlight that the exact mechanisms underlying the disease remain unclear. The development of IBD is believed to result from complex interactions between the host's immune system and both commensal and pathogenic bacteria in the gut. Additionally, it has been suggested that chronic inflammation in IBD is driven by aggressive T-cell responses targeting pathogenic bacteria in the colon (Matricon, Barnich, & Ardid, 2010).

The role of the gut microbiome in IBD has gained considerable attention. Dysbiosis, characterized by an altered microbial composition, is thought to contribute to intestinal inflammation. Imbalance in gut bacteria can lead to immune system activation, which plays a critical role in the pathogenesis of IBD.

Prevalence and Distribution of IBD in the Current Study

In the present study, eleven consecutive patients with IBD were enrolled over a six month period. All patients were diagnosed with Ulcerative Colitis (UC), and none were diagnosed with Crohn's Disease (CD). According to a report by the IBD Task Force established by the Government of India, UC is more prevalent than CD in the Indian population. The task force, which analyzed 1,159 questionnaires, found a UC to CD ratio of 750:409. Notably, in the Western states of India, the UC to CD ratio was 50:9, indicating that UC is four times more common than CD in this region (Ray, 2016).

Total Bacterial Count in IBD Patients Exacerbation vs.Remission Stages

Studies examining total bacterial count via real-time PCR have shown that bacterial load in healthy individuals varies geographically. Most of these studies have used fecal samples to assess the bacterial count in normal individuals (Hopkins, Macfarlane, Furrie, Fite, & Macfarlane, 2005). For instance, the average bacterial load in the gut microbiota of normal individuals in developed countries has been reported as 1.00×10^{11}/ml (Sender, Fuchs, & Milo, 2016), whereas in the Asian population, it is typically 3.57×10^{10}/ml (Marathe, Shetty, Lanjekar, Ranade, & Shouche, 2012).

In the current study, the total bacterial count in the non-IBD control group was observed to be 0.9×10^{11}/ml, which is slightly higher compared to the average bacterial load reported in previous studies of normal individuals. Suryavanshi et al. reported a range of 6.1×10^{10} to 6.8×10^{12}/gm from fecal samples of healthy individuals in Western India (Suryavanshi, Bhute, Jadhav, & Bhatia, 2016). Similarly, Jalanka et al. found that fecal bacterial counts from healthy individuals in Finland, the UK, and the Netherlands ranged from 9.7×10^{10} to 1.15×10^{11}/gm (Jalanka et al., 2015). These studies underscore the geographic variation in total bacterial load.In the present study, we focused on biopsy samples to assess the bacterial count in the mucosal microflora of patients with IBD during acute exacerbations. The results showed a significant reduction in bacterial count compared to the non-IBD control group, with an average bacterial count of 3.12×10^{6}/ml. However, during follow-up visits, when patients were in remission, the bacterial count increased to 2.96×10^{7}/ml.This increase in bacterial count during the remission phase suggests partial reconstitution of the microbial flora with treatment. However, the bacterial load in remission still remained lower than the non-IBD control group, indicating that full recovery of the microbiota had not been achieved. A similar trend was reported by Frank et al. (2007), who observed a tenfold decrease in the total bacterial load in patients with UC compared to healthy controls, with a mean bacterial count of 2.70

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$\times 10^9$/ml in UC patients and 3.60×10^{10}/ml in controls.Similarly, Alan W. Walker et al. (2011) quantified total bacterial load in mucosal biopsy samples from 6 CD and 6 UC patients. They reported a bacterial count of 1.00×10^7/ml during the acute phase of UC, which increased to 1.00×10^8/ml during remission. They also observed a tenfold decrease in bacterial count in the acute phase compared to remission.The differences in bacterial counts observed in our study are consistent with these findings. However, there is limited research comparing bacterial load during disease exacerbations and remission in IBD, particularly for acute exacerbations. Our study aims to fill this gap by examining and reporting the differences in bacterial count between the exacerbation and remission phases in UC patients.

CONCLUSION

Despite the uncertainty of the question " Connection between gut microflora and inflammatory bowel disease ", from the findings of current study and records from previous reports it can be stated that dysbiosis or change in gut related bacterial communities could result in acute exacerbation of IBD. A decrease in the level of microbiota which are capable of producing short chain fatty acids (SCFA) in the gut, may contribute towards inducing the intestinal inflammation, and increase in the levels of bacterial communities having deleterious effect on the intestinal tract may further exaggerate the disease. It would be interesting to study replacement of the lost bacterial communities such as Roseburia, Ruminococcus, Faecalibacterium, Lactobacillus, Oscillospira, Dialister, Megasphaera, and Prevotella with prebiotics & probiotics whether or not it would improve the prognosis in patients of IBD.

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