University of Kerbela

College of Science

Department of Biology

# Investigation Effect of *Escherichia coli* Producing Microcin B17 Isolated from Patients with Inflammatory Bowel Disease in Female Rats

**A Thesis**

Submitted to the Council of the College of the Science / University of Kerbala in Partial Fulfilment of the Requirements for the Degree of Doctorate of Philosophy in Biology

By

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وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ﴾

صدق الله العظيم

(سورة المجادلة- الآية ١١)

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# Dedication

**This thesis is dedicated to my family, whose support and encouragement**

**To my husband, whose understanding and support**

**To my children, whose patience and understanding have been a constant source of motivation throughout this academic journey.**

**This work stands as a testament to the invaluable support of my mother, brothers, and sisters which has been a cornerstone of my academic achievements.**

**Huda/2024**

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**Huda/2024**

# Summary

Inflammatory bowel disease (IBD) describes conditions characterized by chronic inflammation in the gastrointestinal tract, such as Crohn's disease (CD)and ulcerative colitis(UC). In the present study 60 stool samples were collected from patients suffering from IBD, which include 30 samples of CD and 30 samples of UC patients in different age groups and both sexes, these patients were admitted to the Digestive and Liver Diseases Center in Kerbala City government from August 2021 to September 2022. Furthermore, 30 stool samples were collected from healthy individuals as a control. All patients were in active gastroenteritis which was confirmed by testing the fecal calprotectin (FC).

The sex distribution of the patients with IBD showed a higher prevalence of females than males with 60 % versus 40 %. However, the ages of patients with IBD appear to be significantly different, the results indicated that CD is the subtype of IBD that was prevalent among young patients, whereas UC is the subtype that was most prevalent among elderly patients. The most common symptoms were diarrhea in both diseases with blood in stool more prevalent in UC patients. In addition, extraintestinal manifestations may occur such as arthritis, and skin and eye disorders.

*Escherichia coli* was the most prevalent bacterial species in IBD patient's stool, these bacteria have been shown to have many virulence factors including the production of toxins such as microcin B17 and colibactin which recent studies had linked between them and severe gut inflammatory responses.

*E. coli* was isolated from the stool samples by using traditional identification and cultural characteristics ways. The identification was confirmed by PCR technique. However, conventional PCR was used to detect two toxin genes that may have a role in IBD pathogenicity, colibactin and microcinB17. The amplification of the colibactin gene (*clbB*) of 60 isolates of IBD patients (CD and UC) were10 isolates only, (4 from CD and 6 from UC), while the amplification of the toxin microcin B17 ( *McbA*) gene for *E. coli* isolates showed high prevalence in 52 out of 60 isolates (27 isolates from CD patients and 25 isolates from UC patients).

For evaluating the role of microcinB17 in the pathogenicity of IBD, *in vivo*, experiments were done by using twenty-five female rats, which were divided into five groups: the first group, was orally given normal saline, as a control, the second group was orally given *E. coli* with microcinB17 gene ( *E. coli*/+ *McbA*) isolated from the stool of UC patients, the third group rats were orally given *E. coli*/+ *McbA* isolated from the stool of CD patients, the fourth group was orally given *E. coli* negative for microcin B17 gene (*E. coli*/- *McbA*) isolated from the stool of UC patients and the fifth group was orally given *E. coli* negative for microcin B17 gene (*E. coli*/- *McbA*) isolated from the stool of CD patients.

After 15 weeks of administration, and the appearance of some symptoms such as a decrease in weight and a reduction of appetite, blood was drawn for serum serological tests, and the rats were sacrificed to evaluate histological changes in their intestine. The serological changes observed included increased IL-10 and TNF-α levels in their sera. Regarding the histological examination, there were noticeable

histological changes in the large intestines of all rats given bacterial suspension but not in the small intestine.

In conclusion, All *E.coli* isolates which were isolated from IBD patients' stool (whether it was microcin B17 producing or not) were able to induce inflammatory responses and histological changes in the intestines of rats. Accordingly, other virulence factors (in addition to Microcin B17) may play a role in the pathogenesis of IBD.

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## List of Abbreviations

| Abbreviation | Meaning |
|---|---|
| 5-ASA | 5-aminosalicylic acid |
| ADA | Adalimumab |
| ALEC | Adherent and invasive *E. coli* |
| APCs | Antigen-Presenting Cells |
| ASIA | Anti-Saccharomyces cerevisiae antibody |
| CAMs | Cell adhesion molecules |
| CBC | Complete blood count |
| CD | Crohn's disease |
| CFU | Colony-forming units |
| CRC | Colorectal cancer |
| CRP | C-reactive protein |
| CSS | Corticosteroids |
| DAEC | Diffusely adherent *E. coli* |
| DNA | Deoxyribonucleic acid |
| EAEC | Enteroaggregative *E. coli* |
| EGD | Esophagogastroduodenoscopy |
| EIEC | Enteroinvasive *E. coli* |
| EIMs | Extraintestinal manifestations |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EMB | Eosin Methylene Blue |

| | |
|---|---|
| EPEC | Enteropathogenic *E. coli* |
| ETEC | Enterotoxigenic *E. coli* |
| ExPEC | Extraintestinal pathogenic *E. coli* |
| FC | Fecal Calprotectin |
| FMT | Fecal microbiota transplantation |
| GI | Gastrointestinal tract |
| HGB | Hemoglobin |
| IBD | Inflammatory Bowel Disease |
| IFN- γ | Interferon-gamma |
| IFX | Infliximab |
| IL | Interleukin |
| KIA | Kligler iron agar |
| LPS | Lipopolysaccharide |
| MAMPs | Microbe-associated molecular patterns |
| MSI | Microsatellite instability |
| MTX | Methotrexate |
| OD | Optical density |
| p-ANCA | Peri-nuclear antineutrophil cytoplasmic antibody |
| PCR | Polymerase chain reaction |
| RBC | Red blood corpuscle |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| S1P | Sphingosine-1-Phosphate Receptor |
| SCFAs | Short-chain fatty acids |
| STEC | Shiga toxin-producing *E. coli* |

| | |
|---|---|
| Th | T-helper |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor-alpha |
| TPs | Hiopurines |
| UC | Ulcerative colitis |
| UK | United Kingdom |
| UPEC | Uropathogenic *E. coli* |
| VCE | Video capsule endoscopy |
| WBC | White blood cell |

# *Introduction*

# Introduction

Inflammatory Bowel Disease (IBD) is a chronic gastrointestinal disease, Crohn's disease (CD) and ulcerative colitis (UC) are two distinct kinds of IBD (Flynn & Eisenstein,2019). Despite their similarities, they differ in their pathological mechanisms. Both diseases are more often diagnosed in urban areas compared to rural areas and both have their challenges. It will become a serious health problem in the near future, even in developing countries (Seyedian *et al.*,2019). Given that the prevalence of this disease is higher at younger ages it affects half of the patient's life. The etiology of IBD is unknown, but studies demonstrated genetics, immunology, diet, microbiome, and other environmental variables as potential reasons (Flynn & Eisenstein,2019).

The inflammation is triggered in IBD patients as a result of either excessive effector T-cell function or deficient regulatory T-cell function, associated with the overproduction of pro-inflammatory cytokines, such as Tumor-necrosis factor (TNF) or a deficiency in the production or function of regulatory/immunosuppressive cytokines such as IL-10 (Leppkes & Neurath,2020).

*Escherichia coli* (*E. coli*) is a well-studied member of the Enterobacteriaceae family; in fact, multiple investigations have discovered an increased incidence of virulent *E. coli* strains recovered from IBD patients compared to healthy controls ( Baldlli *et al.*,2021). *E. coli* isolates resemble extraintestinal pathogenic *E. coli* (ExPEC) rather than the conventional diarrheagenic pathotypes which contain several virulence factors, as well as the production of particular bacteriocin determinants (Micenková *et al.*,2018).

These bacteria may play an important role in initiating and maintaining the inflammatory process in IBD patients' gut tissues by providing antigens or other stimulatory factors that activate the immune system. (Mirsepasi-Lauridsen *et al*.,2019).

Colibactin is a secondary metabolite produced by many Enterobacteriaceae, including pathogenic *E. coli*, and is typically found in the mucosal tissue of individuals with inflammatory bowel disease and colorectal cancer. In cell lines and pre-clinical models, *E. coli* with this toxin causes DNA damage and cancer development (Dougherty &Jobin,2021). Colibactin crosslinks the DNA by activating the SOS signaling pathway. These distinct activities of colibactin highlight its ecological role in changing gut microbial communities and its consequences on human health (Mousa,2022). On another hand, microcinB17 is a toxin produced by the same family (Enterobacteriaceae) specifically *E. coli* which recent research has linked to the development of inflammatory bowel disease (IBD). microbial toxin microcin B17 can directly trigger gastrointestinal inflammation by modifying the activity of CD1d, a major histocompatibility complex (MHC) molecule. CD1d is found in intestinal epithelial cells (IECs) and hematopoietic cells, and functions to present lipid antigens to natural killer T cells (NKT) (Iyer *et al*.,2018; Collin & Maxwell,2019).

Animal models are essential for determining mechanistic insights that will allow for better preclinical settings to target specific factors involved in IBD etiology, as well as, in the screening and development of new drugs for their treatment, which would be impossible in humans (Mizoguchi *et al*.,2020).

# CHAPTER I

The study aims to evaluate the role of *E. coli* with the microcinB17 gene in the development of IBD in animal models.

The aim is achieved through the following practical steps:

1. Isolation and identification of *E. coli* from the stool samples of IBD patients (Both Crohn's disease and ulcerative colitis) and healthy individuals as a control group.
2. Detection of the presence of colibactin and micricinB17 genes in isolated bacteria by Polymerase Chain Reaction technique.
3. Pathological study of *E. coli* with and without microcinB17 gene in the rat's intestine.
4. Serological and histological study of administered rats with bacteria and compare them with control rats.

# *Literature Review*

## 1-1 Inflammatory Bowel Disease: Crohn's Disease and Ulcerative Colitis, Definition and Etiology

Inflammatory Bowel Disease (IBD), is a gastrointestinal disorder that clinically includes Crohn's disease(CD) and ulcerative colitis(UC). IBD is characterized by periods of stomach pain, diarrhea, bloody stools, weight loss, and an influx of neutrophils and macrophages that cause inflammation and ulceration. IBD affects both males and females at a young age and has been recognized as one of the most common gastrointestinal disorders with an accelerated incidence in newly industrialized countries since the beginning of the 21st century. (Guan,2019). CD typically affects any part of the intestine in a patchy manner. In contrast, UC affects the rectum and can spread continuously across the entire colon or only a portion of it. The inflammation in UC is only present in the mucosa and submucosa with cryptitis and crypt abscesses, in contrast to the thickened submucosa, transmural inflammation, fissuring ulceration, and granulomas that are histologically present in CD ( Hoter & Naim, 2019) Figure 1-1.

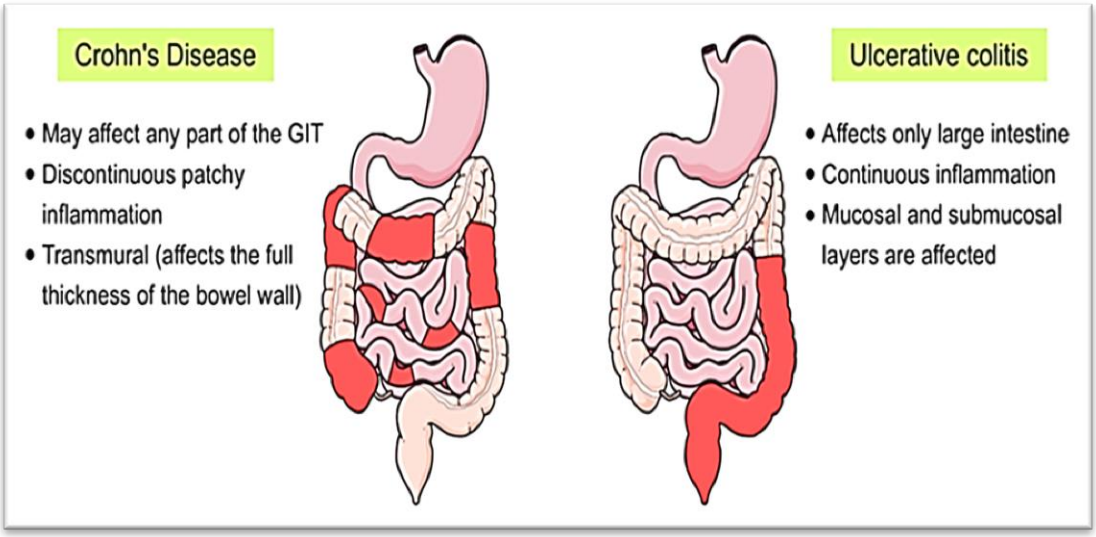



**Figure 1-1: The main differences between the two main forms of inflammatory bowel disease (IBD) (Hoter & Naim, 2019)**

## 1-2 The Epidemiology of IBD

Inflammatory Bowel Disease (IBD) is a global disease. However, In industrialized countries (North America, Europe, and Oceania) IBD was heralded by the Industrial Revolution in the 1700s: manufacturing, increased air pollutants, dietary changes such as less plant-based fibers,

and societal shifts from rural to urban living. Abercrombie's book 1828 describes several cases in the United Kingdom (UK) that, retrospectively, are thought to have been some of the earliest recorded cases of IBD (Kaplan & Ng, 2016). From those first few confirmed cases of IBD in the UK, the disease exploded through the population during the latter half of the twentieth century (Soon *et al*.,2012). Despite the stabilization in incidence, the Western world is experiencing compounding prevalence, which is observed in diseases with low mortality, a young age of onset, and no current-known cure; with a steady influx of new cases and relatively few annual deaths in the afflicted population (Kaplan,2015).

In contrast, the incidence of IBD is rising in many newly industrialized countries in Asia and Latin America. The rise in incidence is believed to be secondary to the Westernization of cultures and societies in these regions (Windsor & Kaplan,2019). In the mid-to-late twentieth century, the typical Chinese diet had begun to resemble a Western diet: increased amounts of animal and dairy products, refined sugars, processed foods, and fewer plant-based fibers. China underwent an industrial revolution in the 1950s. Not coincidentally, the first documented case of UC in China was in 1956 (Kaplan & Ng, 2016). By 2000, there were approximately 2000 individuals in China with CD and 10,000 individuals with UC; by 2010, 266,394 people in China were estimated to have IBD (Kaplan & Ng, 2016). Similar to the trends, observed in China, there were familiar patterns of disease penetration in other countries around the world.

The rising prevalence of IBD over the last century and a half is a substantial concern for medical practitioners: namely, an aging population presents unique challenges for disease management due to the longer disease duration and a greater risk of comorbid conditions (Nguyen *et al*.,2019). Seniors (those aged 65+) are the fastest-growing subpopulation of people with IBD due to both an aging population of previously diagnosed cases and newly diagnosed elderly-onset cases (Benchimol *et al*.,2019). In contrast, the rate of early-onset IBD, affecting people < 18 years old, has been rapidly increasing throughout the twenty-first century: a Canadian study found that between 1999 and 2010, the number of pediatric IBD patients increased by 50% (Benchimol *et al*.,2017).

Based on historical patterns, Coward *et al*.(2019) project reported that the prevalent population of IBD patients in North America will nearly double in the next 12 years, from approximately 2 million to almost 4 million. Beyond the almost doubling of the prevalent population, healthcare systems will need to manage the complications of the fastest-growing subpopulation of IBD patients.

## 1-3 The Etiology of IBD

The causes of IBD are not fully understood, gut inflammation may be caused by a combination of genetic, environmental, microbial, and host-related factors (Flynn & Eisenstein,2019). These factors play a significant role in promoting intestinal inflammation, primarily through their impacts on the microbiome composition. However, for microbial dysbiosis to cause uncontrolled intestinal inflammation, the intestinal barrier formed by intestinal epithelial cells as well as the innate immune system must be affected. The immune system activation is dependent on the

working balance of effector and regulatory cells in the intestinal mucosa, which has also been found to be downregulated in the patient population. As a result, IBD pathogenesis is caused by the interaction of genetic susceptibility and environmental impact on the microbiome, which leads to inappropriate intestinal immune activation via a weakened intestinal barrier (Ramos & Papadakis,2019). The possible causes of IBD can be illustrated in Figure 1-2.

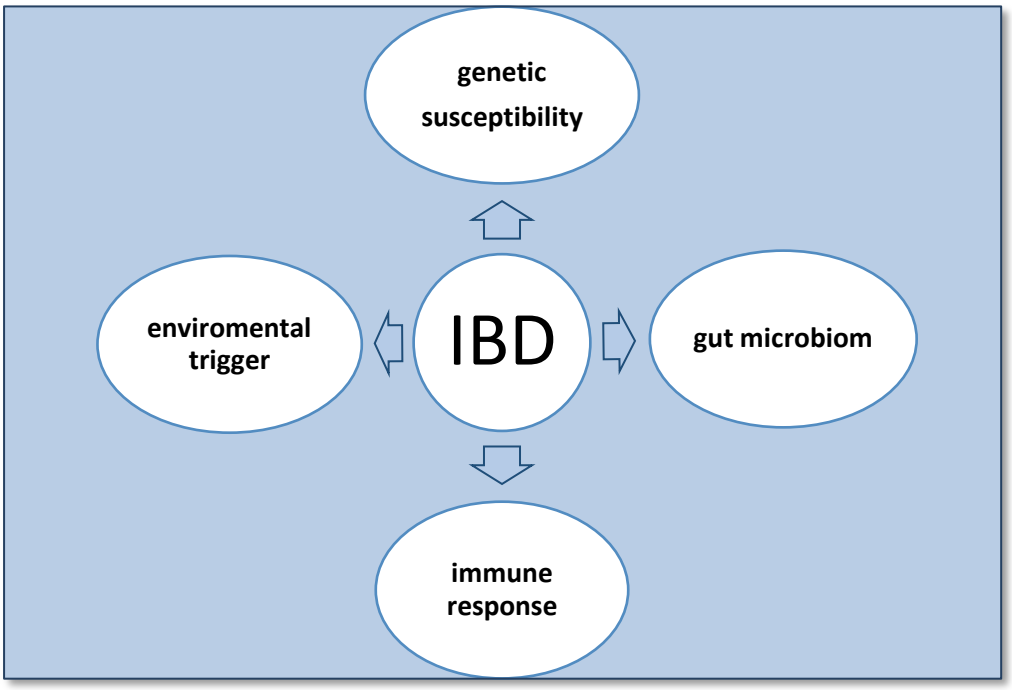



**Figure 1-2: The possible causes of IBD (Flynn & Eisenstein,2019)**

### 1-3-1 Genetic Susceptibility

Population-based studies have offered strong evidence that genetic factors play a role in the pathogenesis of IBD, they illustrated an 8- to 10-fold increased risk of IBD among family members of UC and CD probands; and, most importantly, they demonstrated twin concordance. Overall, the significantly higher concordance for CD vs UC in monozygotic twins suggests that genetic factors contribute to CD more than UC (Cho & Brant, 2011).

Inflammatory Bowel Disease has been associated with more than 201 mutations, and genome-wide association analyses suggest that IBD is most likely a polygenic condition. 41 CD-specific genes such as *SPRED1*and *NOD2*, and 30 UC-specific genes such as *TNFRSF14* and *RFTN2* have been identified, although 137 loci are associated with both CD and UC together such as *IL1R1*, *IFIH1*, and *IL18RAP*. (Flynn & Eisenstein , 2019).These genes can be grouped into three categories operating separately on distinct checkpoints of the inflammatory cascade; including mutation in genes that are related to (1) infection identification, (2) pathogen clearance by

innate and cell-mediated immunity, and (3) delay of pathogen invasion across the intestinal mucosal barrier (Younis *et al*.,2020).

**1-3-2 Gut Microbiota**

Microbe-host interaction is critical for the maintenance of vital host functions, and its disruption has been linked to several diseases including IBD. Commensal microbiota play an important role in the early development of the immune system, and their products regulate immune responses by inducing immune cells, signaling pathways, and inflammatory mediators ( Santana *et al*.,2022)

The study of microbial pathogenesis of IBD has primarily focused on three areas: 1) The theory of the persistent pathogen 2) the theory of excessive bacterial translocation; and 3) the theory of dysbiosis. According to the persistent pathogen theory, IBD can be caused by long-term infection with an enteric pathogen such as *Mycobacterium avium* subspecies paratuberculosis, *Clostridium difficile*, or pathogenic *Escherichia coli*; according to the theory of excessive bacterial translocation, IBD occurs on by a high level of intestinal bacteria crossing the intestinal barrier; while the dysbiosis theory proposed that IBD can be caused by a shift in the balance of "useful" vs. "harmful" commensal bacteria ( Liu *et al*.,2021).

**1-3-3 Immune Response**

The development of IBD is associated with the host adaptive immune response, both CD and UC cause an increase in T cell-mediated responses. In CD, Inflammation begins to occur by an elevated T-helper Th1 and Th17 response, which causes the release of proinflammatory cytokines such as interleukin (IL)-17, interferon-gamma (IFN- γ), and tumor necrosis factor-alpha (TNF-α), leading a self-perpetuating cycle of inflammation. In UC, the response is Th2-mediated, leading to more effective activation of B cells and natural killer T cells via IL-5 and IL-13 (Ramos & Papadakis,2019).

The intestinal barriers are closely linked to the host's innate immunity, the environment is made up of intestinal epithelial cells (enterocytes, goblet cells, neuroendothelial cells, Paneth cells, and M cells). Disturbances of this barrier are revealed to cause IBD in animal models, such as Muc2-deficient mice, which cannot release mucin from goblet cells and can develop IBD on their own (Van der Sluis *et al*.,2006). Paneth cell deficiencies, which have been associated with CD development, are mediated by NOD2 gene abnormalities. NOD2 has been demonstrated to be important in autophagy and intracellular pathogen clearance, and its disruption has been connected to the development of CD (Fritz *et al*.,2011). The development of IBD is also linked to Antigen-Presenting Cells (APCs) in the mucosal barrier in which CD patients have lower macrophage activity allowing for more bacterial shifting across the gut ( Ramos & Papadakis,2019).

### 1-3-4 Environmental Trigger

Dietary factors are linked to the development of IBD in various populations. Saturated fat and processed meat diets have been linked to the development of disease. A study by Hou JK *et al*., (2011) demonstrated that high-fiber diets reduce the risk of CD. Medications that disrupt the host microbiome (antibiotics, nonsteroidal anti-inflammatory drugs, contraception, and statins) have all been linked to an increased risk of IBD development ( Ramos & Papadakis,2019). Besides, data support an association between smoking and CD whereas smoking cessation, but not current smoking, is associated with an increased risk of UC, depression impaired sleep, and low vitamin D levels have all been associated with incidents of IBD (Ananthakrishnan, 2015).

## 1-4 The Role of Microbiota in IBD:

The human microbiota is made up of over 100 trillion microbial cells, the majority of which are bacteria found in the gastrointestinal tract. Because many microbiota-derived species contribute to host physiology, the interaction between these bacteria and the host is usually symbiotic. Maintaining intestinal flora balance and preventing the proliferation of potentially harmful species is therefore crucial in disease prevention. Changes in the bacterial composition of the gut can promote persistent inflammation and the formation of gastrointestinal diseases including inflammatory bowel disease (IBD) (Baldelli *et al*.,2021; Gevers *et al*.,2014).

Pathologic changes in the gastrointestinal (GI) microbiota have been shown in a recent study to activate a mucosal immune response, resulting in chronic intestinal inflammation (Liu *et al*.,2021) Dysbiosis is caused by changes in the GI microbiota, as a result, disturbance of intestinal eubiotic state in IBDs can be regarded as a cause rather than a consequence of chronic inflammation in the GI. (Hansen & Sartor,2015).

Numerous studies have found that the GI microbiota is involved in the pathogenesis of IBD (Knights *et al*.,2014). Typical dysbiosis is frequently observed in CD and UC patients (Manichanh *et al*.,2012). IBD patients, in particular, have a reduction in the amount of protecting commensal anaerobic bacteria such as *Faecalibacterium prausnitzii*, Clostridium spp., and *Bacteroidetes frag*ilis. These bacteria have anti-inflammatory properties, including the ability to modify the host's mucosal autoimmune interaction through the production of short-chain fatty acids (SCFAs)like acetate, propionate, and butyrate are a primary source of energy for colonic epithelial cells. As a result, these bacteria are not adequately represented in IBD, and the beneficial effects of SCFAs, such as the inhibition of pro-inflammatory cytokine expression, the production of mucin and antimicrobial peptides, and tight junction protein are downregulated and reduced (Sokol *et al*.,2009). Figure 1-3

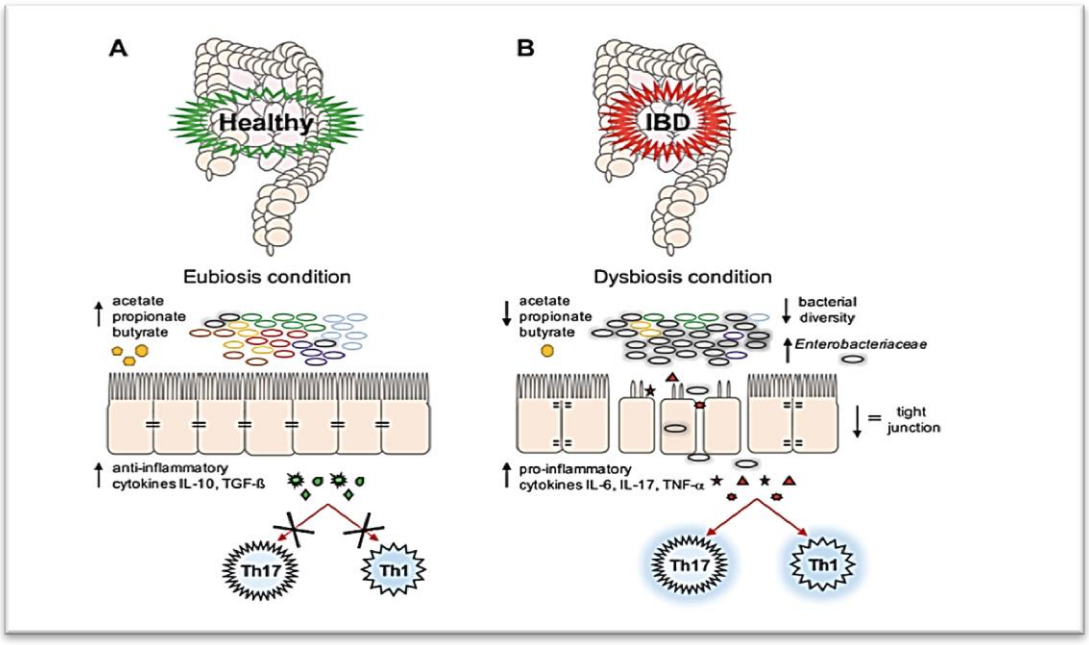



**Figure 1-3: A diagram of the eubiosis (A) and dysbiosis (B)consequences. (Baldelli *et al*.,2021)**

Many studies have reported an increase in the proportion of potentially harmful Proteobacteria, especially the Enterobacteriaceae family, in IBD patients. Therefore, it would appear that the host's inflammatory response could be the trigger of gut microbiota imbalance, most likely caused by Enterobacteriaceae blooming, which leads to the persistence of IBD's inflammatory state (Baldelli *et al*.,2021). Enterobacteriaceae molecular elements directly promote the inflammatory response such as microbe-associated molecular patterns (MAMPs), which are compounds observed on bacteria's surfaces. These chemicals generate inflammation by interacting with immune cell receptors. ( Scales *et al*.,2016). The mechanisms by which Enterobacteriaceae bloom in IBD, are not completely known. To explain this observation, the "oxygen hypothesis" has been formulated (Rigottier-Gois,2013). In physiological conditions, enterocytes reduce oxygen levels in the gut lumen by beta-oxidation processes, creating an anaerobic environment. In IBD patients, beta-oxidation is reduced, and the oxygen level is increased. This event leads to an increase in facultative aerobes such as Proteobacteria/Enterobacteriaceae and, as a consequence, intestinal dysbiosis ( Hughes *et al*.,2017) or through the mechanism of competition and bacterial fitness by producing the microcins (Markovic *et al*.,2022)

## 1.5 The Role of *Escherichia coli* in IBD

*Escherichia coli* (*E. coli*) is a Gram-negative bacterium that colonizes the intestinal tract of infants shortly after birth and contributes to maintaining normal intestinal homeostasis. *E. coli* strains are divided into three major groups based on genetic and clinical criteria: Commensal strains found in the human and animal gut (lacking specialized virulence factors), intestinal

pathogenic strains (diarrheagenic), and extraintestinal pathogenic *E. coli* (ExPEC) ( Mirsepasi-Lauridsen *et al*.,2019).

The six well-known intestinal pathogenic strains (diarrheagenic) *E. coli* types include Enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely adherent *E. coli* (DAEC). The gastrointestinal diseases caused by these *E. coli* strains range from self-limiting diarrhea to hemorrhagic colitis ( Mirsepasi-Lauridsen *et al*.,2019).

Several bacteriological analyses of fecal microbiota in IBD patients have revealed that a diverse range of bacterial species, including *E. coli*, is more prevalent in the stool of IBD patients (Pascal *et al*.,2017; Eaves-Pyles *et al*.,2008).

Inflammatory bowel disease *E. coli* isolates have been shown to differ from healthy *E. coli* isolates, by representing a heterogeneous population that was more similar to extraintestinal pathogenic *E. coli* (ExPEC) than to the classic diarrheagenic pathotypes (Micenkova *et al*., 2018). The majority of IBD *E. coli* strains belonged to phylogenetic groups B2 and D, which are common in ExPEC strains ( Vejborg *et al*., 2011; Kotlowski *et al*.,2007). *E. coli* in inflammatory bowel disease patients have distinct metabolic capabilities that enable colonization of intestinal mucosa ( Fang *et al*.,2018).

The adherent invasive *E. coli*(AIEC) pathotype, on the other hand, was identified by Darfeuille-Michaud *et al*.(2004) about several years ago. It had distinct phenotypic pathogenic traits and was linked to CD but not UC.

The members of AIEC can adhere to and invade epithelial cells via a process involving (i) actin microfilaments and microtubule recruitment; (ii) surviving within the macrophage phagolysosome; (iii) inducing the production of tumor necrosis factor (TNF) from infected macrophages; and (iv) adhesion to and invasion of epithelial cell lines (Palmela *et al*.,2018; Tyakht *et al*.,2018; Chervy *et al*.,2020). A study by Buisson *et al*., (2022) supported the idea that AIECs are involved in the early steps of ileal CD. High prevalence of *E. coli* isolated from ileal biopsy specimens from CD patients; 100% in early lesions and 65% in chronic lesions. These findings suggested that *E. coli* may play a role in both the initiation and the chronic promotion of inflammatory processes in CD.

Another study by Meheissen *et al*.,(2019) demonstrates that Enteroaggregative *E. coli* (EAEC) was identified in 25% of IBD patients.

The differences between studies could be based on the type of the samples that were analyzed or on the disease severity that influences the distribution of phylogroups in IBD patients, as an increased proportion of B2 and D isolates have been found in active IBD patients ( Sepehri *et al.,*2011).

Schussler *et al*.(1976) discovered a significant increase in antibody titers against the lipid A and O antigens of *E. coli* in CD, UC patient groups, and acute enteritis groups compared to healthy controls, and suggested that these titers could be used to distinguish CD from UC. The polyclonal antibodies against *E. coli* were found in macrophages within the lamina propria,

indicating that inflammation was the first step. Furthermore, antibodies against the *E. coli* outer membrane protein C were detected in 55% of IBD patients (Mow *et al*.,2004).

## 1.6 Colibactin Toxin:

Colibactin is a secondary metabolite encoded by the *pks* gene island, that has been identified in numerous Enterobacteriaceae, including some pathogenic *E. coli*, and is frequently detected in mucosal tissue from individuals with inflammatory bowel disease and colorectal cancer. *E. coli* carrying this biosynthetic gene cluster causes DNA damage and tumorigenesis in cell lines and pre-clinical models ( Dougherty & Jobin,2021). The colibactin structure is composed of two approximately symmetrical subunits, each of which contains an electrophilic cyclo-propane warhead that binds to adenine residues on DNA to form interstrand cross-links Figure 1-4. Recent studies have provided the first insight into the molecular structure of colibactin and identified DNA-reactive electrophilic sites that are capable of alkylating DNA by ring-opening addition (Dougherty & Jacobin,2021).

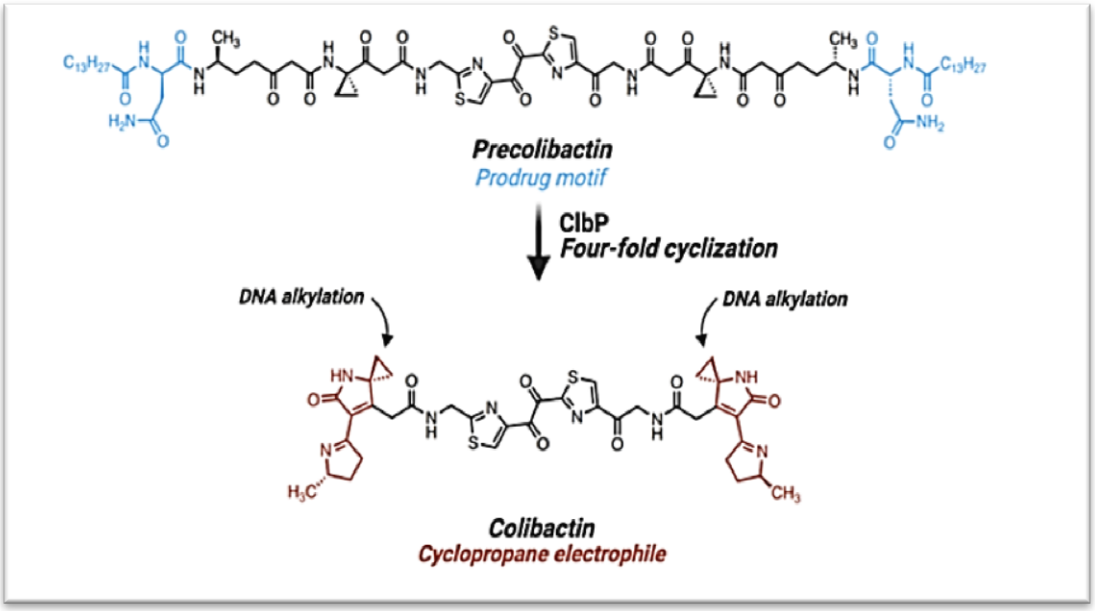



**Figure 1- 4: The structure of colibactin ( Dougherty & Jobin,2021).**

Colibactin not just targets the eukaryotic cell machinery but also the prokaryotic cell colibactin crosslinks the DNA resulting in activating the SOS signaling system, and this leads to the prophage induction from bacterial lysogens and modification of virulence genes in pathogenic species. These distinct activities of colibactin highlight its ecological functions in altering gut microbial ecosystems, as well as, the effects on human health (Mousa,2022).

Accumulating evidence indicates that *E. coli-producing* colibactin in IBD patients is inducing the activity of epithelial oxygenation, decreasing mucosal barrier integrity, and promoting bacterial biofilm formation (Tang-Fichaux *et al*.,2021). Furthermore, patients with IBD, have a higher lifetime risk of developing colorectal cancer (CRC) due to higher levels of colibactin exposure and the direct result of years of chronic inflammation (Lakatos & Lakatos,2008). Additionally, many studies have described the prevalence of colibactin and its ability to cause epithelial DNA damage in human cases of IBD and CRC (Arthur *et al*., 2012; Bonnet *et al*., 2014; Prorok-Hamon *et al*., 2014).

## 1-7 Microcin B17 Toxin

Microcin B17 was first isolated from *E. coli* strains, and its expression was later linked to the plasmid pMccB17, which carries the mcb operon which is illustrated in Figure 1-5. This operon contains seven genes: The structural gene *mcbA* encodes a peptide that is post-translationally modified by the microcin synthases (*mcbB, mcbC, mcbD*), which convert serine and cysteine residues within the peptide sequence into oxazole and thiazole rings. The remaining genes, *mcbE, mcbF*, and *mcbG*, code for proteins involved in export and immunity (Collin *et al*., 2013).

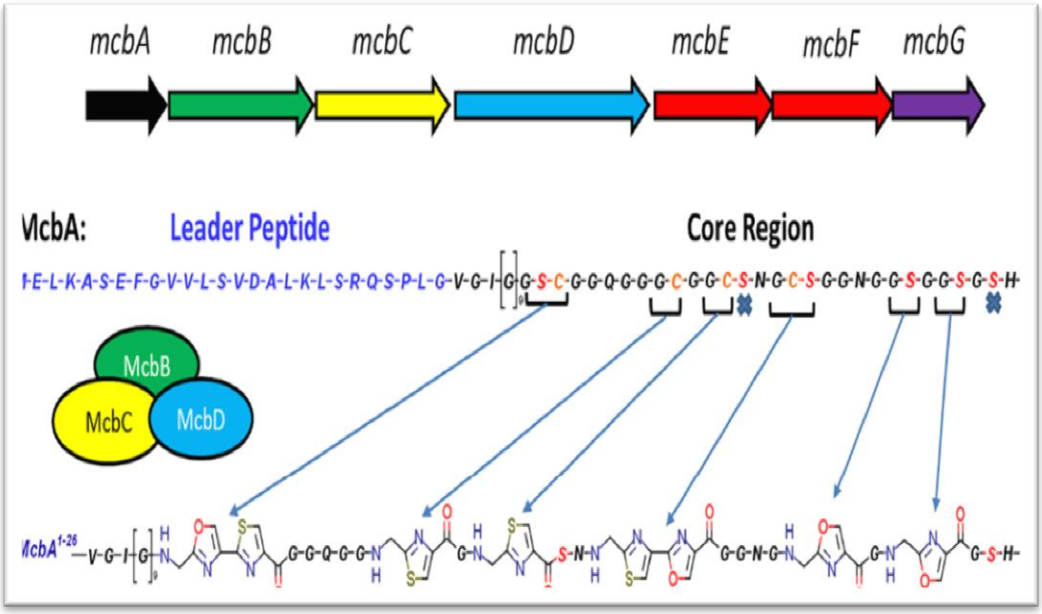



**Figure 1-5: The structure of microcin B17(Collin & Maxwell,2019)**

Microcin B17 are low molecular mass bacteriocins produced by *E. coli* under stress conditions as a mechanism for bacterial fitness (Markovic *et al.*,2022). MicrocinB17 was initially identified by its ability to inhibit bacterial DNA replication leading to the induction of the SOS system, DNA degradation, and cell death (Herrero & Moreno, 1986). When incubated in a cell-free extract, Microcin B17 induces double-strand cleavage of DNA and inhibits DNA gyrase by binding to it trapping it in an intermediate cleavage complex which leads to the accumulation of double-stranded genomic DNA breaks, and eventual death (Withanage *et al*.,2023; Collin *et al*.,2019; Collin *et al*., 2013).

Although a major motivation for studying MicrocinB17 has been the hope that its mode of action can be exploited to develop new antibiotics, most work on this toxin has been fundamental. Microcin B17 is an inhibitor with relatively modest activity on gyrase-like quinolones, able to stabilize a transient gyrase–DNA covalent complex, an event that leads to the generation of double-strand DNA breaks that are lethal to the cell. The exact mechanism of microcin B17 action is still unknown. MicrocinB17 is not an easily druggable molecule; however, its action needs many factors (Collin & Maxwell,2019). Therefore, it was surprising when a link between MicrocinB17 and inflammatory bowel disease (IBD) was recently reported (Iyer *et al*.,2018; Collin & Maxwell,2019).

*In vivo*, B17 microcin derivatives may promote intestinal inflammation via the mechanism of CD1d-dependent pathology, as well as induction of both Th1 and Th2 cytokines in the colon of experimental animals by intrarectal administration (Iyer *et al*.,2018).

## 1.8 Diagnosis of the Inflammatory Bowel Disease

To diagnose IBD, the clinical symptoms of the disease must be investigated. Clinical symptoms include pediatric growth disorders anemia, abdominal pain, bloody diarrhea, and arthritis. On the other hand, precise tests are required to diagnose CD and UC (Seyedian *et al*.,2019). The important laboratory tests are:

**1-8-1 Serologic Markers:** The antibodies that are useful in differentiating IBD subtypes, including peri-nuclear antineutrophil cytoplasmic antibody (p-ANCA), which has a strong link with ulcerative colitis, and Anti-Saccharomyces cerevisiae antibody (ASCA), which exists in approximately 60% of CD patients, have shown their specificity as well as sensitivity in diagnosing CD (Flynn& Eisenstein, 2019).

**1-8-2 Inflammatory Markers**:

Numerous biomarkers have been identified to aid in the monitoring of the inflammatory process. C-reactive protein (CRP), for example, is the most sensitive blood sign for inflammation. Inflammation damages tissue, causing macrophages to generate a cytokine cascade that ends in CRP production in the liver. Within hours following the initial insult, CRP levels can rise dramatically, reaching 500- to 1000-fold greater than baseline. Because of its short half-life of 19 hours, CRP is an interesting biomarker for inflammation; nevertheless, it is general, and its levels can rise as a response to a wide range of tissue damage (Flynn& Eisenstein, 2019).

Fecal Calprotectin (FC) is a direct marker of mucosal inflammation in the intestine. This protein is produced by neutrophil degranulation and is derived from the cytosol of neutrophils. As a result, its levels can be raised in situations of diverticulitis, infectious colitis, intestinal tumors, and cirrhosis. FC is stable in feces samples for up to 7 days. FC's utility as a diagnostic tool is defined by both the positive value and the pretest probability for IBD. Clinicians believe that an FC level of 50 to 100 mg/g indicates stable disease, a level of 100 to 200 mg/g is indeterminate, and a level of 250 mg/g shows active inflammation. (Flynn& Eisenstein, 2019).

Both CRP and FC have been used as treatment response markers. They may, however, respond directly to the therapy even if the inflammation has not subsided (Bernstein *et al.*,2016).

### 1-8-3 Endoscopy and Biopsy :

The endoscopy is critical in the diagnosis, management, and treatment of IBD. Endoscopy is essential for controlling other causes, establishing diagnoses, distinguishing CD from UC, monitoring disease activity and response to treatment, and evaluating and treating complications. Endoscopy can be classified into several types (Spiceland &Lodhia,2018), including:

1. The lower endoscopy (colonoscopy, flexible sigmoidoscopy): A colonoscopy with intubation and investigation of the terminal ileum should be performed as part of the initial evaluation of patients with clinical manifestations suggestive of IBD. Colonoscopy combined with ileoscopy not only allows for direct visual examination of the colon and terminal ileum but also allows for biopsies. When IBD is suspected, two biopsy specimens from five sites, including the ileum and rectum, are preferred. Biopsies of both affected and normal-looking mucosa should be taken. The combination of endoscopic and histologic features aids in the diagnosis of IBD by distinguishing CD from UC and ruling out other disease entities with similar presentations. Colonoscopy is a safe procedure with few side effects in IBD patients. However, patients with severe colitis and toxic megacolon may be at risk. In cases where a full colonoscopy is not appropriate, flexible sigmoidoscopy, which allows for distal bowel examination and biopsy collection, can be a safer option. In addition, in patients with established IBD, flexible sigmoidoscopy can be used to assess disease severity and/or rule out infection.
2. The upper endoscopy or esophagogastroduodenoscopy (EGD): Due to the overlap of IBD symptoms and upper endoscopy indications, such as abdominal pain, weight loss, nausea, and vomiting, EGD is frequently used in the diagnostic evaluation of suspected IBD. Furthermore, in adults with unclassified IBD, upper endoscopy can aid in the diagnosis of CD. EGD is recommended as part of the initial evaluation of children with suspected IBD, regardless of upper GI symptoms. Upper endoscopy can also be used to screen for celiac disease, which can present in adults and children similarly to IBD.
3. The wireless video capsule endoscopy (VCE): Since its approval in 2001, it has evolved into a safe and effective small intestine imaging technology. Early mucosal lesions are more sensitive to detection than radiologic studies. A significant limitation of VCE is the inability to obtain tissue for histologic diagnosis.

## 1-9 Histopathology of IBD Colitis

Both CD and UC differ from each other in epidemiological aspects, clinical presentation, endoscopic and histopathological findings, and disease course; the two entities have different complications and management (Loddenkemper, 2009). However, the histopathological findings on biopsy samples can overlap between the two entities. Thus, for a correct interpretation of biopsy specimens from patients with suspected IBD, detailed clinical information is essential to make a correct differential diagnosis and to schedule appropriate therapy and follow-up (Langner *et al*.,2014). On the other hand, a correct pathological approach, both on adequate biopsy samples and surgical specimens is useful to better recognize histopathological findings of IBD, subclassify UC or CD, and exclude non-IBD colitis.

The histological diagnosis of IBD is based upon the assessment of four main features: (I) mucosal architecture, (II) lamina propria and submucosal cellularity, (III) neutrophil granulocyte infiltration, and (IV) epithelial abnormality (Magro *et al.,*2013). Thus, knowledge of the normal histology of GI-mucosa is essential for optimal interpretation of biopsy specimens.

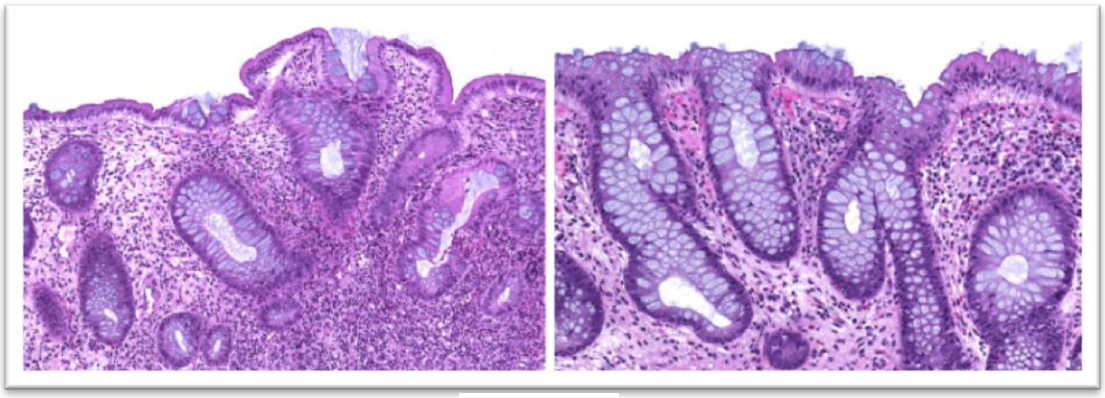
Table 1-1

**(I)** The morphologic features of mucosal architecture: changes are termed crypt architectural distortion. Normal crypts are straight, parallel, and extend from the surface to nearly above the muscularis mucosae. The crypt architectural distortion observed in IBD is characterized by irregularly arranged, dilated, branched, and/or shortened crypts. This can be a sign of ongoing inflammation or the process of regeneration (Langner *et al*.,2014).

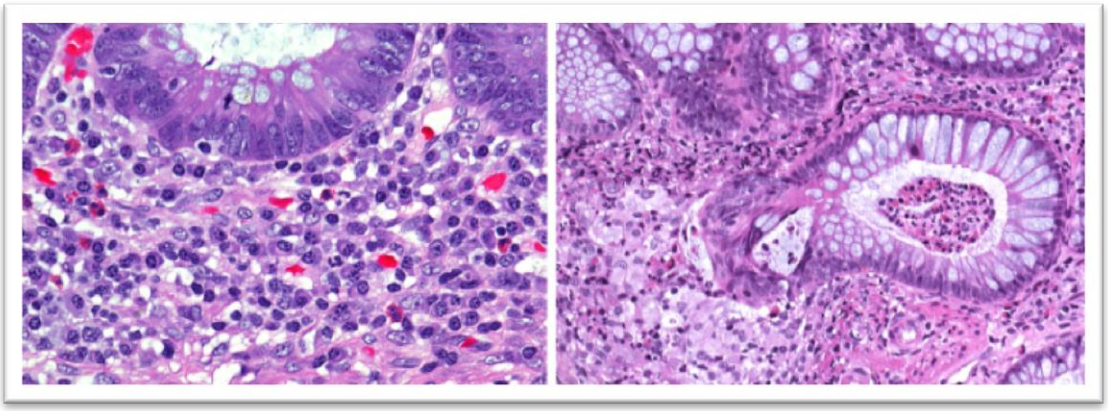
**(II)** The abnormal cellularity of the lamina propria: refers to an increased and altered distribution of cell types that are normally present. Lymphocytes and plasma cells are always found in the colorectal lamina propria. In the normal colon, the number of inflammatory cells varies depending on the anatomical site. The abundance of eosinophil granulocytes varies a lot between normal individuals (Magro *et al*.,2020).

**(III)** The neutrophil granulocyte infiltration: is the hallmark of active disease. Neutrophils can be found in the lamina propria or they can invade the crypt surface epithelium (termed cryptitis) and the lumen of crypts forming crypt abscesses. Neutrophils are in general not found in normal mucosa (Langner *et al*.,2014).

**(IV)** The epithelial abnormalities found in IBD include mucin depletion, metaplastic changes, and surface epithelial damage. Mucin depletion can be defined as a decreased number of goblet cells or a decreased amount of intracellular mucin. Metaplastic changes are seen in the form of pyloric gland metaplasia (a typical sign of chronicity in ileal involvement of CD or Paneth cell metaplasia. The damage of the surface epithelium, e.g., focal cell loss, flattening, erosions, and ulcers reflects the activity of the disease. (Magro *et al.,*2013).



A



B

**Figure 1-6: Marked crypt architectural distortion(cryptitis) and hypocellular stroma in quiescent disease (A), Plasma cells are predominantly observed between the base of the crypts and the muscularis mucosae ('basal plasmacytosis') (B) (Langner *et al*.,2014).**

Inflammatory activity is defined by the presence of neutrophils. Neutrophilic cryptitis, crypt abscesses, hemorrhage, erosions, ulceration, and necrosis are all features of active inflammation. The pathology report should describe the histological features of chronicity and activity and preferably also grade the degree of activity as mild/moderate/severe.

**Table 1-1: Microscopic findings useful for the diagnosis of inflammatory bowel disease (Kellermann & Riis, 2021)**

| Characteristic findings | Ulcerative colitis | Crohn's disease |
|---|---|---|
| Inflammation involvement | Limited to mucosa, or | Often transmural |

| | | |
|---|---|---|
| | superficial submucosa | |
| Chronic inflammation distribution | Diffuse, often most marked distally | Focal, patchy |
| Crypt architectural distortion | Often marked | Present, but often less marked |
| Neutrophil granulocytic infiltration | Continuous | Focal, patchy |
| Crypt abscesses | Typical | Less typical |
| Lymphoid aggregates | Often in basal mucosa | Often transmural |
| Histiocytic granulomas | Only about ruptured crypts | Typical |

## 1-10 The Improvement of Intestinal Microecology:

Changes in the composition and function of the intestinal microbiota were found in patients with IBD. Although the specific mechanism of IBD remains unclear, the occurrence of IBD is found closely related to the imbalance of intestinal micro-ecology(Ocansey *et al*.,2019). The improvement of intestinal micro-ecology can be done by the following:

### 1. Probiotics, Prebiotics and Synbiotics:

- Probiotics are live microorganisms that are intended to have health benefits when consumed or applied to the body such as bacteria that produce lactic acid.
- Prebiotics can be health-promoting substrates selectively utilized by host microbes and supplemented by the intake of legumes, fruits, and vegetables including polyols (sugar alcohols), oligosaccharides, and soluble fiber.
- Synbiotics are the synergistic combination of probiotics and prebiotics found in foods, drugs, and supplements ( Abraham & Quigley,2020), figure(1-7).

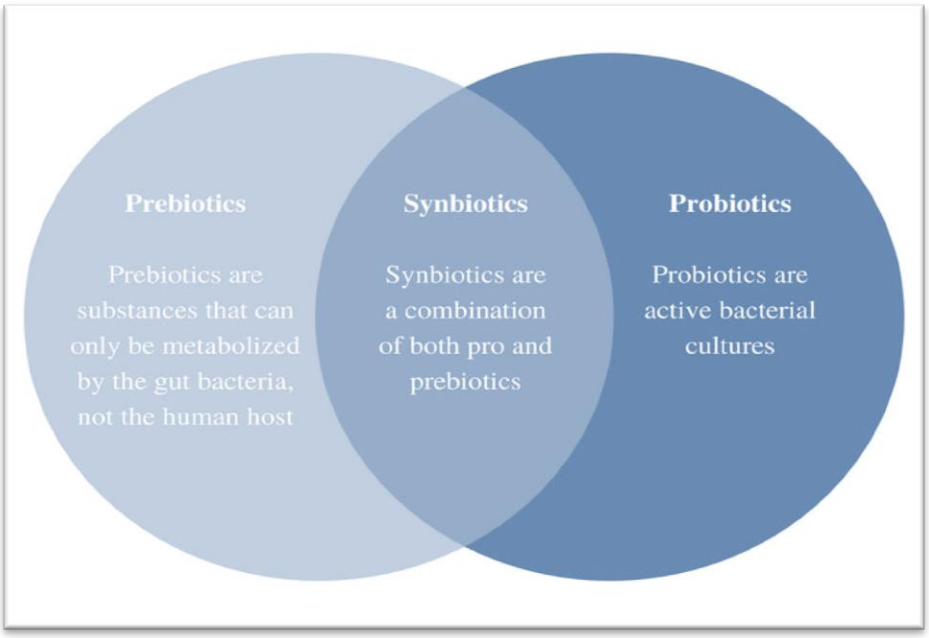



**Figure 1-7: Probiotics, Prebiotics and Synbiotics supplements ( Abraham & Quigley,2020)**

2. **Fecal microbiota transplant (FMT)**: FMT is a new therapy that transplants the functional microbiota from the feces of healthy donors into the gastrointestinal tract of patients suffering from intestinal microbiome disorders to reconstruct the intestinal microecology and cure disease. FMT is effective in the treatment of recurrent and refractory CDI with a high success rate of 90% ( Grehan *et al.*,2010; Quraishi *et al.*,2017; Jeon *et al.*,2018).

## 1-11 Colorectal Cancer in Inflammatory Bowel Disease

Patients with inflammatory bowel disease (IBD) are at significantly increased risk of colorectal cancer (CRC), principally resulting from the pro-neoplastic effects of chronic intestinal inflammation. However, the incidence has declined over the past 30 years, attributed to both successful CRC surveillance programs and improved control of mucosal inflammation (Keller *et al.*,2019).

Risk factors that further increase the risk of IBD-related CRC include:

- **Disease duration:** CRC risk begins to increase 8–10 years after the diagnosis is made, and increases over time. The incidence rates corresponded to cumulative probabilities for IBD-CRC of 2% by 10 years, 8% by 20 years, and 18% by 30 years (Eaden *et al*.,2001).
- **Extent and severity:** Pancolitis patients are at higher risk, with prevalence of 5.7%. Patients with left-sided UC and patients with penetrating disease and who have had immunosuppressive therapy in CD are also at significantly higher risk of IBD-CRC (Scaringi *et al*.,2013).
- **The presence of inflammatory pseudopolyps:** Pseudopolyps, representative of healed severe mucosal damage, and prior severe inflammation double the risk of CRC within the affected region (Velayos *et al*.,2006).
- **Family history of CRC:** Family history is associated with increased risk. IBD patients with CRC in a first-degree relative have twice the risk of developing CRC than those who do not (Askling *et al*.,2001).

Molecular alterations that occur in sporadic CRC, such as chromosomal instability, microsatellite instability (MSI), and hypermethylation, play a role in IBD-CRC, but the order and frequency of these mutations, and the fact that they often occur before definite histologically defined dysplasia, differentiates IBD-CRC from sporadic CRC (Sebastian *et al*.,2014). IBD patients tend to have excessive inflammatory cell infiltration and expression of several inflammatory genes; this mucosal inflammation promotes cellular proliferation and ultimately CRC development (Velayos *et al*.,2006; Lakatos & Lakatos, 2008).

The dysbiosis of gut microbiota appears to have a role in IBD-CRC development (Kang& Martin,2017). *E. coli* a major contributor to the induction of chronic inflammation during IBD and convertor of IBD into CRC is dramatically increased in IBD. The lipopolysaccharides of the gram-negative bacteria increase the expression of TLR4, a common event during IBD-CRC tumorigenesis (Kang *et al*.,2013). *E. coli* also activates NF-kB expression, which plays a role in inducing inflammation and CRC development. Colibactin-equipped *E. coli* is more prevalent in

the colonic mucosa in CD and UC patients (Cuevas-Ramos *et al*.,2010; Arthur *et al*.,2012; Bonnet *et al*.,2014).

# *Materials & Methods*

# 2-1 Materials

## 2-1-1 Equipment and Apparatus:

The equipment and apparatus used throughout the study are listed in Table 2-1:

**Table 2-1: Equipment and apparatus used in the present study**

| Apparatus | Manufacture Company(origin) |
|---|---|
| Autoclave | Lab Tech (Korea) |
| CBC (Complete Blood Count) apparatus | Mindray(China) |
| Centrifuge | Jrenetzki (Germany) |
| Distillation | GFL (Germany) |
| Gel electrophoresis Unit | ThermoScientific (USA) |
| Hot Plate Magnetic Stirrer | Ikama Ret (India) |
| Incubator | Memmert (Germany) |
| Light Microscope | Olympus (Japan) |
| Master thermocycler gradient (PCR) | BioRad (USA) |
| Microcentrifuge | ThermoScientific (USA) |
| Microtiter Plate (ELISA) Reader | Biotech (USA) |
| Microtome | Leica (Germany) |
| Nanodrop system | Thermo-scientific (USA) |
| Oven | Memmert (Germany) |
| Refrigerator | Philips (Holland) |
| Sensitive balance | Sartorius (Germany) |
| Spin centrifuge | Benchmark (USA) |
| UV transilluminator documentation system | Major Science (Taiwan) |
| Vortex | Fanem (Brazil) |
| Water Bath | Memmert (Germany) |

## 2-1-2 Chemicals

The chemicals used in the study are summarized in Table 2-2

**Table 2-2: Chemicals and solutions were used in the present study**

| Chemicals and Solutions | Manufacture Company(origin) |
|---|---|
| Absolute ethanol | GCC (England) |
| Agarose | Promega (USA) |
| Barium chloride (BaCl2.2H2O) | BDH (England) |
| Canada Balsam | Schuchardt Manchen (Germany) |
| Chloroform | Scharlau (Spain) |
| Deionized Distilled Water | Promega (USA) |
| DNA Ladder 100-1200pb | Promega (USA) |
| DNA Loading dye | Promega (USA) |
| Eosin | BDH (England) |
| Ethidium bromide | Promega (USA) |
| Formalin | GCC (England) |
| Glycerol | BDH (England) |
| Gram stain Kit | SYRBIO(Syria) |
| Green Master Mix | Promega (USA) |
| Hematoxylin crystals | BDH (England) |
| Hydrogen Peroxide ($H_2O_2$) 3% | BDH (England) |
| N, N, N, N-tetramethyl-P-phenylene diamine dihydrochloride | BDH (England) |
| Normal Saline (Sterile) | PSI (Saudi Arabia) |
| Paraffin Wax | Wollen weber (Germany) |
| Primers | Scientific Research Co. (Iraq) |
| Sulphuric acid ($H_2SO_4$) | GCC (Germany) |
| TBE buffer | Promega (USA) |
| Tris-Borate-EDTA(TBE) Buffer (10X) | Promega (USA) |
| Xylene | BDH (England) |

## 2-1-3 Kits Used in This Study

The Kits used in this study are illustrated in Table 2-3

**Table 2-3: Kits used in this study**

| Kits | Manufacture Company(origin) |
|---|---|
| Calprotectin detection kit | BIOTEC (Spain) |
| Rat Interleukin 10 | BT LAB (China) |
| Rat Tumor necrosis factor-α | SUNLONG(China) |
| Genomic DNA purification kit | Promega (USA) |

## 2-1-4 Ready to Use Culture Media

Ready to use culture media used in this study are depicted in Table 2-4:

**Table 2-4: Ready to use culture media used in the study**

| Culture media | Manufacture Company(origin) |
|---|---|
| Brain heart infusion agar | Himedia (India) |
| Brain heart infusion broth | Himedia (India) |
| Eosin Methylene Blue agar | Himedia (India) |
| Kligler iron agar | Himedia (India) |
| MacConkey agar | Oxoid (England) |
| Nutrient agar | Himedia (India) |
| Nutrient broth | Himedia (India) |

Following the manufacturer's instructions, all ready-to-use culture media was prepared. The pH was adjusted and sanitized by autoclaving at 121ºC for 15 min. before being distributed into sterile Petri dishes and stored at 4 ºC until use.

## 2-1-5 Solutions, Reagents and Stains

**2-1-5-1 McFarland Standard Solution (tube No. 0.5) (MacFaddin, 2000)**

It is the turbidity standard solution which is the most widely used method of inoculum preparation or standardization especially the McFarland 0.5 standard, which is prepared by adding 0.5ml of a 1% (w/v) solution of barium chloride ($BaCl_2.H_2O$) and 99.5ml of a 1% (v/v) solution of pure sulphuric acid ($H_2SO_4$) to obtain barium sulfate solution with the specific optical density to provide turbidity comparable to that of bacterial suspension containing $1.5\times10^8$ CFU/ml. The tube was stored in the dark at room temperature (20-25ºC). Standards were stored for six months.

**2-1-5-2 Reagents**

A) **Catalase Reagent**:

This reagent is composed of 3% hydrogen peroxide ($H_2O_2$) (Forbes *et al*., 2007)

B) **Oxidase Reagent:**

It was prepared freshly with 0.1g of N, N, N, N-tetramethyl-P-phenylene diamine dihydrochloride was dissolved in 10 distilled water, stored in a dark bottle, and used immediately (Heritage *et al*., 2003).

**2-1-5-3 Gram Stain:**

Gram stain consists of crystal violet, iodine, ethanol (95%), and safranin (Prescott *et al*., 2004).

## 2-1-6 Detection of Calprotectin Production

All the stool samples were tested for calprotectin production, by using the CERTEST Calprotectin 50+200 (µg/mg) kit which consists of two parts:

1. Stool sample collection tube for preparation and dilute the sample.
2. Calprotectin comb card test which contains two strips 50 + 200 (µg/mg)

## 2-1-7 Materials Used in DNA Extraction, Agarose Gel Electrophoresis, and PCR Amplification

**2-1-7-1 Genomic DNA purification kit** (Promega, USA)

This system contains sufficient reagents for 100 samples of extraction of genomic DNA from *E. coli* bacteria **It contains solutions in Table 2-5:**

**Table 2-5: Solution provided in the extraction of genomic DNA kit**

| components | Quantity determination) |
|---|---|
| Solution of Cell Lysis | 100 ml |
| Solution of Nuclei Lysis | 50 ml |
| Solution of Protein Precipitation | 50 ml |
| Solution of DNA Rehydration | 50 ml |
| Solution of RNase | 250 µl |

- At room temperature (25ºC) the kit was maintained.

**2-1-7-2 Ethyl Alcohol (70%):** It was made by placing 70.1ml of absolute ethanol (99.9%) into a volumetric flask and diluting it to 100ml.

**2-1-7-3 Ethidium Bromide Dye**

It was prepared at a final concentration of 10 mg/ml by dissolving 0.1g of ethidium bromide in 10 ml distilled water in a dark bottle, stirred with a magnetic stirrer until completely dissolving then kept at 4ºC (Maniatis *et al*., 1982).

**2-1-7-4 Agarose Gel (1%)**

A concentration of 1% agarose was used in DNA electrophoresis after extraction. It was prepared by dissolving 1g of agarose in 100 ml of (1X) TBE buffer, heated on a hot plate until boiling; after that, cooled to 50ºC (Sambrook & Russell, 2005).

## 2-1-8 Materials and Solution Used in ELISA Technique for Detection Rat's Serum IL-10 and TNF-α

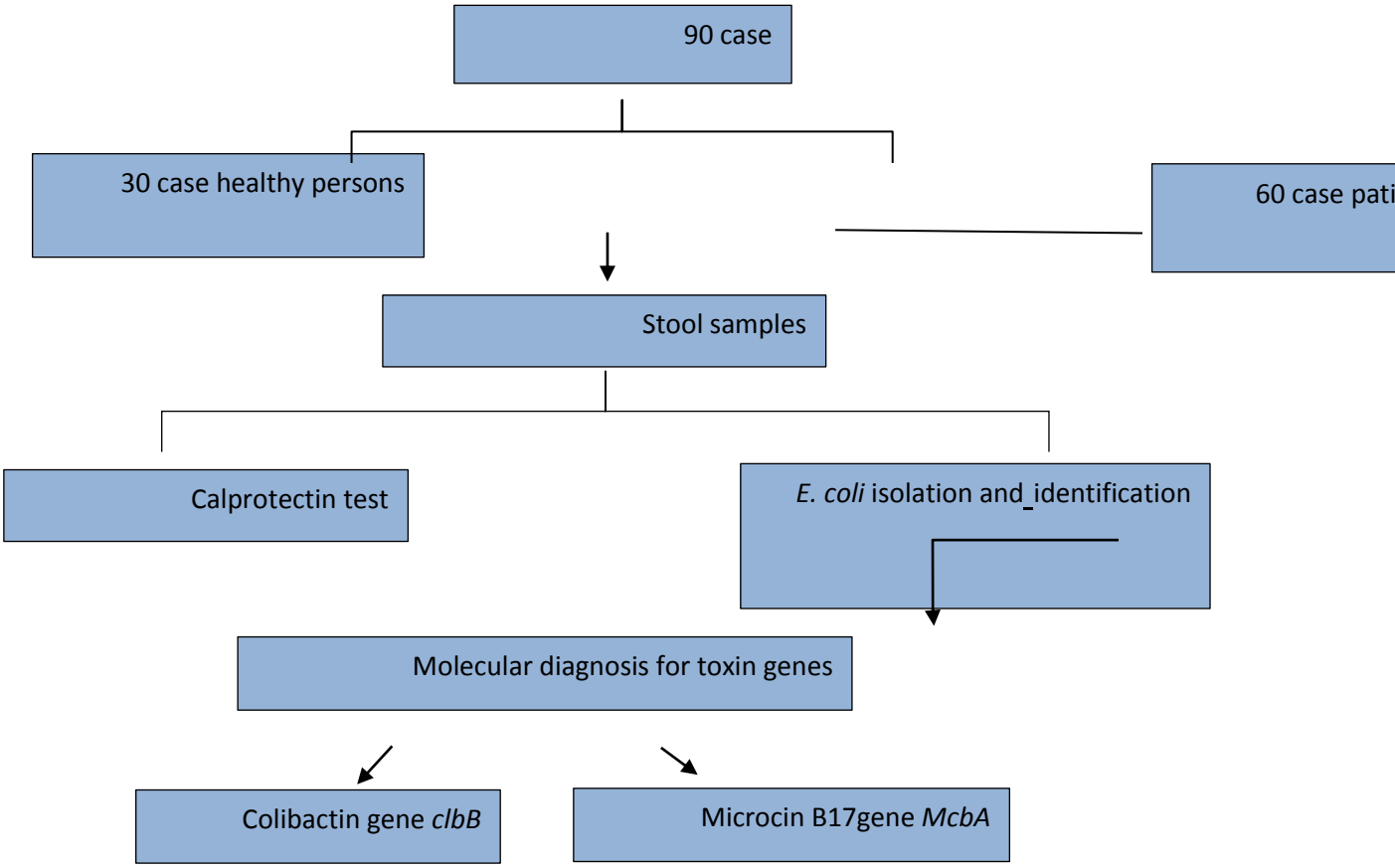
These materials are illustrated in Table 2-6

**Table 2-6: Materials and solutions provided with the kit of IL-10 and TNF-α**

| components | Quantity determination) |
|---|---|
| Standard solution (960pg/ml) | 0.5ml x1 |
| Pre-coated ELISA plate | 12 * 4 well strips x1 |
| Standard diluent | 3ml x1 |
| Streptavidin-HRP | 3ml x1 |
| Stop solution | 3ml x1 |

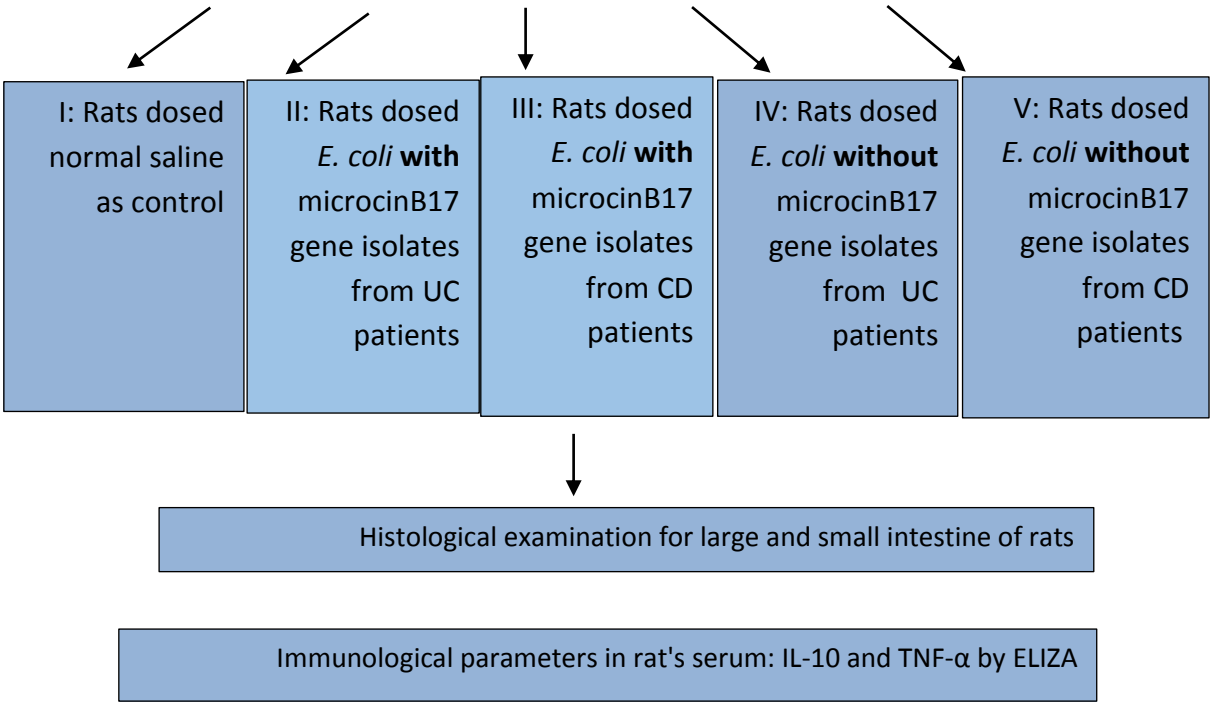
| | |
|---|---|
| Substrate solution A | 3ml x1 |
| Substrate solution B | 3ml x1 |
| Wash buffer Concentrate (25x) | 20ml x1 |
| Biotinylated Rat IL-10 | 1ml x1 |

## 2.2 Study Design: Section I




## Section II

Orally administration of rats which divided 5 groups




## 2-3 Methods:

## 2-3-1 Stool Specimen's Collection:

**A) Patients:** Sixty stool samples were collected from patients aged between 5 to 70 years old from both sexes suffering from IBD who attended the Center of Digestive Tract and Liver Diseases in Karbala City during the period from August 2021 to September 2022. Furthermore, information was taken from them through a questionnaire paper that included questions about their age, important symptoms, side manifestations, and the effects of some diets. Appendix (1).

**B) Control**: Thirty samples were collected from healthy people as controls in different ages and sexes. Fecal samples were transported in a sterile container to the laboratory.

## 2-3-2 Detection of Calprotectin Production:

According to the instruction of the manufacturing company, the diluted stool sample was put in the comb card test which contains two strips 50 + 200 (µg/mg) as follows:

1- The stick was used to pick up the sample from the cap.
2- The stool sample was put into the diluent tube and shaken to ensure good sample dispersion.

3- The end of the tube was cut and dispensed exactly 4 drops into each comb card to test the 50 and 200 windows.

4- The color in the bands appeared and the results were read later than 10 minutes. If calprotectin concentration was less than 50 (µg/mg) it might mean neither active gastrointestinal inflammation nor the risk of relapse (CD or UC) if the result was between 50-200 (µg/mg) it might mean active gastrointestinal inflammation and if the result more than 200 (µg/mg) they had a cute inflammation. The figure of the procedure was illustrated in Appendix (2).

## 2-3-3 Isolation and Identification of *Escherichia coli*

The collected specimens were first prepared by picking a little stool 1 gram by a stick and diluting it with 19 ml of normal saline, then the diluted stool streaked directly on MacConkey agar, and incubated for 24 hours at 37ºC. Lactose fermenting colonies were picked and recultured on fresh MacConkey agar plates to obtain pure well-isolated colonies. The obtained colonies were streaked on Eosin Methylene Blue to investigate the appearance of green metallic sheen colonies. Further identification tests included the morphological features and biochemical tests carried out depending on Lupindu (2017) Further identification was carried out by PCR method to confirm the identity by using the amplification of *16s rRNA* gene. Table 2-7.

## 2-3-4 Identification of *Escherichia coli* isolates

The isolates were identified depending on the morphological features, cultural characteristics, and biochemical tests according to Bergy's Manual of Systematic Bacteriology, 2nd edition (Garrity *et al*., 2005). PCR technique was employed to confirm the identification.

### 2-3-4-1 Microscopic examinations

All isolates were stained by Gram stain to detect their response to stain, and shapes, and their arrangement was observed by light microscope.

### 2-3-4-2 Cultural characteristics

#### A) Growth on MacConkey agar

MacConkey agar was used for testing the isolate's shape, size, color, consistency, and lactose fermentation.

## B) Growth on Eosin Methylene Blue agar

Eosin Methylene Blue agar plates were used to detect the ability of each isolate to form green metallic sheen colonies.

### 2-3-4-3 Biochemical tests

All biochemical tests were carried out according to Forbes *et al*. (2007) unless mentioned elsewhere.

## A) Catalase test

One to two drops of catalase reagent (3% $H_2O_2$) were placed on a slide and an amount of pure bacterial growth was transferred by a wooden stick and mixed with the reagent on the slide. The positive results were indicated by air bubble formation.

B) **Oxidase test**

A piece of filter paper was placed in a clean Petri dish and 2-3 drops of freshly prepared oxidase reagent (1% N-N-N-N-tetramethyle-p-phenylene diamine dihydrochloride) were added to the filter paper. A colony from the tested bacterium was transferred to the filter paper and rubbed onto the reagent with a sterile wooden applicator stick. The positive result was indicated by blue-purple color formation within 10-15secs.

C. **Kligler Iron Test**

Kligler iron agar slant prepared as mentioned in 2-1-4 was inoculated with tested bacterial culture by streaking on the surface and stabbing in the bottom of the medium, incubated at 37°C for 24hrs, The positive result was noticed by changing the color of the phenol red indicator from red to yellow as a result for acid production from sugar fermentation, cracks, and bubbles appear at the stabbing place as a result of gas production from sugar aerobic fermentation, black residue appeared at the butt of the tube as a result for the reaction between $H_2S$ produced from anaerobic fermentation with ferrous sulfate in the medium. While the negative result observed by keeping the phenol red indicator its red color, bubbles, and black residue don't form. This test was used for the differentiation of members of the Enterobacteriaceae based on the fermentation of lactose, glucose, and the production of $H_2S$.

## 2-3-5 Preservation of Isolates

Preservation of isolates was performed as follows:

**2-3-5-1 Short Time Preservation:**

The *E. coli* isolates were grown on 5ml of nutrient agar in the screw-capped tubes, as slants; they were tightly tied with parafilm and kept at 4ºC for three months (Harely and Prescott, 2002).

**2-3-5-2 Long-Term Preservation:**

For more than three months the bacteria were stored in a nutrient broth mixed with 20% glycerol without considerable loss of viability at low temperatures. It was done by adding 0.2ml of sterilized glycerol to on exponential growth of bacteria in a screw-capped tube of nutrient broth with a final volume of 1ml and stored at -20ºC for months (Vandeppitte *et al.,* 2003).

## 2-3-6 DNA Extraction from *Escherichia coli*:

According to the instruction of the manufacturing company, DNA was extracted and purified from all *E. coli* isolates using a commercial purification system (Wizard genomic DNA purification kit, Promega, USA). DNA was extracted according to the bacterial protocol procedure of Gram-negative bacteria as follows:

1) Isolates of *E. coli* were activated by culturing them in a nutrient broth at 37ºC for 24 hours. After that, 1 ml of bacterial growth was transported into a microcentrifuge tube. The cells were centrifuged at 13000 rpm for 2mins and the supernatant was removed.

2) Six hundred microliters of Nuclei Lysis Solution were added and gently pipetted until the cells were re-suspended. The tube was incubated in a water bath at 80ºC for 5 minutes to lyse the cells, and then it was cooled to room temperature.

3) Three microliters of RNase were put into the cell lysate and 2-5 times inverted to mix, at 37ºC incubated for 45mins and then the tubes were cooled at room temperature.

4) Two hundred microliters of Protein Precipitation were added to the cell lysate RNase-treated and vigorously vortexed at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate, then centrifuged at 13000 rpm for 3mins.

5) The DNA-containing supernatant was transferred to a clean 1.5ml microcentrifuge tube containing 600 µl at room temperature alcohol.

6) The tube was then gently mixed until the DNA-like thread-strands formed a visible, at 13000 rpm for 2 mins centrifuges. The supernatant was carefully poured away and the tube was drained on clean absorbent paper.

7) Six hundred microliters of 70% ethanol were added and the tube was gently inverted several times to wash the DNA pellet, centrifuged at 13000 rpm for 2mins, and ethanol was aspirated carefully. The tube was drained on clean absorbent paper and the pellet was allowed to air-dry at room temperature for 10-15mins.

8) One hundred microliters of DNA Rehydration Solution were added to the tube and rehydrated by incubating the solution overnight at 4ºC. The resultant DNA was stored at - 20 ºC.

## 2-3-7 Measurement of Concentration and Purity of Extracted DNA

After DNA extraction, 2µl of DNA was transferred into a Nanodrop instrument for checking of concentration and purity of extracted DNA samples at 260 nm and 280 nm wavelengths, the TBE buffer was used as blank. The $OD_{260}$ reading was considered as the concentration of DNA while the $OD_{260/280}$ ratio reading was considered as the purity of DNA.

## 2-3-8 Polymerase Chain Reaction (PCR) Technique

The PCR assay was performed in a monopole pattern to amplify different fragments of genes under study in *E. coli* isolates.

### 2-3-8-1 Primers Selection and Preparation

The primers listed in Table 2-7 were selected for this study for PCR technique; these primers were provided in a lyophilized form, dissolved in sterile nuclease-free water as recommended by the manufacturing company to give a final concentration of 100 pmol/µL then diluted to 10 pmol/µL concentration by adding nuclease-free water and stored in deep freezer to be used later.

Gradient PCR was used to determine an optimal annealing temperature for the genes which was used in this study using the least number of steps. This optimization was achieved in one experiment. Gradient PCR provides a gradient function that in one single run evaluates up to 12 different annealing, elongation, or denaturation temperatures ( Achyar *et al.*,2021). Table 2-8, 2-9, 2-10.

**Table 2-7: The primers used in this study**

| Gene name | Primer sequence 5'-3' | Amplicon size (bp) | References |
|---|---|---|---|
| *16S* -rRNA | F-CATGCCGCGTGTATGAAG AA | 100 | Lyadorai *et al*.,2020 |
| | R–CGGGTAACGTCAATGAGCAAA | | |
| *McbA* | F-TCCGCCTGCGGAATTAATGA | 203 | This study |
| | R-TACTGATTGCCACCGTCCTG | | |
| *clbB* | F-GCGCATCCTCAA GAGTAAATA | 280 | Lyadorai *et al*.,2020 |
| | R-GCGCTCTATGCTCATCAACC | | |

**2-3-8-2 PCR Amplification Reaction**

The extracted DNA, primers, and PCR green master mix were mixed. The PCR mixture was set up in a total volume of 25µL including 12.5µL of PCR green master mix, 1µL of each primer, and 2µL of template DNA used, and the rest volume was completed to 25µL with sterile nuclease-free water. PCR reaction tubes were spin centrifuged briefly to mix and bring the contents to the bottom of the tubes then vortexed, and finally placed into a thermocycler PCR instrument where DNA was amplified.

**Table 2-8: PCR amplification program for *16-rRNA* gene**

| Stage | Temperature | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 4 min | 1 |
| Denaturation | 95°C | 30 sec | 30 |
| Annealing | 60°C | 30 sec | |
| Extension | 72°C | 1 min | |
| Final extension | 72°C | 8 min | 1 |

**Table 2-9: PCR amplification program for *McbA* gene**

| Stage | Temperature | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 4 min | 1 |
| Denaturation | 95°C | 30 sec | 30 |
| Annealing | 55°C | 30 sec | |

| | | | |
|---|---|---|---|
| Extension | 72°C | 1 min | |
| Final extension | 72°C | 8 min | 1 |

**Table 2-10: PCR amplification program for *clbB* gene**

| Stage | Temperature | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 4 min | 1 |
| Denaturation | 95°C | 30 sec | 30 |
| Annealing | 60°C | 30 sec | |
| Extension | 72°C | 1 min | |
| Final extension | 72°C | 8 min | 1 |

## 2-3-9 DNA Electrophoresis on Agarose Gel

Agarose gel electrophoresis can be used for the detection of genomic DNA fragments and PCR products to analyze the quantity and quality of DNA which is visualized with the aid of an ethidium bromide and UV transilluminator documentation system (Magdeldin, 2012).

### 2-3-9-1 Agarose Preparation

It was performed according to Podzorski *et al*. (2006) method.

A) A concentration of 1% agarose was used for the detection of extracted DNA

B) Aliquot 3µl Ethidium bromide staining solution was added to the agarose solution and mixed well.

C) The pouring plate (tray) of the electrophoresis device was prepared by sticking the tray edges very well by sticking tape to prevent leaking of the gel from the tray and prevent bubbles formation.

D) The comb was fixed at one end of the tray for making wells used for loading DNA samples.

E) The agarose gel was poured into the preparing tray after comb fixing and left to solidify for 30 minutes.

### 2-3-9-2 DNA Sample Loading in Agarose Gel

An aliquot of 7µl of each DNA template or PCR product was transferred to the Eppendorf tube, 3µl of loading dye was added to the tube of genomic DNA and the mixture was loaded into the wells in agarose gel. Later, 5µl of the DNA ladder was loaded in the first well. The electric current was allowed at 75 volts for 1 hour. UV transilluminater was used for the observation of DNA bands and the gel was photographed by a digital camera (Magdeldin, 2012).

## 2-3-10 *In vivo* Pathogenicity Study of *Escherichia coli* Isolates from (CD and UC) Patients in Rats Gastrointestinal System

### 2-3-10-1 Laboratory Animals

Twenty-five female white rats were used, brought from the animal house of the College of Pharmacy - Kerbala University, their ages ranged between (12-14) weeks, and their average weights ranged between (200-210) grams, they were placed in cages prepared for this purpose in the animal house of the College of Pharmacy. Water and food consisting of poultry ration were provided as they were given freely ad libitum under controlled conditions of proper ventilation. At a temperature of (25°C), and a lighting period of 12 hours of light and 12 hours of darkness throughout the experiment, the animals were left to acclimatize for two weeks before the start of the experiment.

### 2-3-10-2 Preparation for Rat's Administration

The bacterial cell suspensions were prepared at $1.5 \times 10^8$ colony-forming units/ mL (CFU/ml) following McFarland turbidity standard N° 0.5. For this purpose, 18 h old overnight bacterial cultures were prepared on brain heart infusion agar, and a few bacteria colonies were collected aseptically with a sterile loop and introduced into 10 ml of sterile 0.90% saline distilled water and homogenized.

### 2-3-10-3 The Experimental Design

This experiment was designed to investigate the effect of *E.coli* containing microcinB17 gene (*E. coli*/+ *McbA*) isolated from patients with inflammatory bowel disease (IBD) on the intestines of rats by oral administration of 1.5 ml of a suspension containing $1.5\times10^8$ CFU of *E. coli* prepared at 0.5 McFarland turbidity scale. The animals were grouped as follows:

1. First group, control group rats were orally given 1.5 ml of normal saline.

2. Second group, rats were orally given 1.5 ml *E. coli*/+ *McbA* isolated from the stool of UC patients.
3. Third group, rats were orally given 1.5 ml *E. coli*/+ *McbA* isolated from the stool of CD patients.
4. Forth group, rats were orally given 1.5 ml *E. coli* that do not harbor the microcin B17 gene (*E. coli*/-*McbA*) isolated from the stool of UC patients.
5. Fifth group, rats were orally given 1.5 ml *E. coli* that do not harbor the microcin B17 gene (*E. coli*/-*McbA*) isolated from the stool of CD patients.

During fifteen weeks and 35 doses to each rat, the weight of the animals was monitored weekly with the appetite and stool texture.

**2-3-10-4 Collecting Blood Samples**

The animals were anesthetized with chloroform and dissected by opening the abdominal cavity. Blood samples were collected in 4 ml from each animal by drawing blood directly from the heart using the heart-puncture method. To obtain the largest amount of blood, blood samples were collected using medical syringes (5 ml). the blood sample was divided into two tubes 2ml in EDTA tubes to assess complete blood account and 2 ml of blood was placed in gel tubes free of anticoagulant to obtain a sufficient amount of serum and left for 15-20 minutes, then the tubes were transferred to a centrifuge at a speed of 3000 revolutions per minute for 15 minutes The serum was obtained and distributed into numerous clean and sterile tubes and kept frozen at -20°C to measure the levels of interleukins – 10 (1L-10) and Tumor Necrosis Factor-α (TNF-α) by using the Enzyme-Linked Immunosorbent Assay (ELISA) technique.

## 2-3-11 Serum Level of Rat Interleukin-10 and Tumor Necrosis Factorα

Sera of rat groups were assessed in 2-3-10-3 for levels of IL-10 and TNF-α by the ELISA method that was based on similar principles for the two cytokines.

**2-3-11-1 The Technique Principle**

This kit is a sandwich ELISA. The plate has been pre-coated with Rat IL-10 or TNF-α antibody (Capture Antibody) and is adsorbed onto wells of a 48-well plate. The antibody present in the sample or standard is added and binds to antibodies coated on the wells. After that, a biotinylated anti-antibody is added and binds to IL-10 or TNF-α captured by the

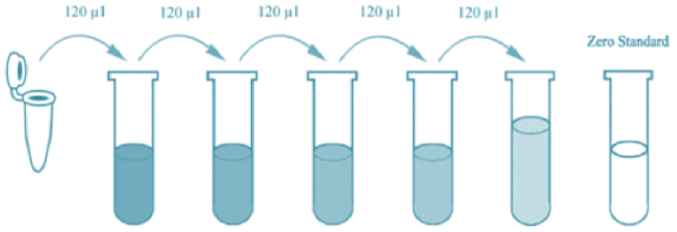
first antibody (Detection Antibody). Following incubation, unbound biotinylated anti-antibody is removed during a wash step. A streptavidin-horseradish peroxidase (HRP) is added and binds to the Biotinylated IL-10 or TNF-α antibody. After incubation, unbound Streptavidin-HRP is washed away during a washing step. A substrate solution is then added and color develops in proportion to the amount of Rat IL-10 and TNF-α. The reaction is terminated by the addition of an acidic stop solution and absorbance is measured at 450 nm.

**2-3-11-2 Reagent Preparation**:

According to the instructions of the manufacturing company, all reagents should be brought to room temperature before use. Reconstitute the 120µl of the standard (960pg/ml) with 120µl of standard diluent to generate a 480pg/ml standard stock solution. The standard was cited for 15 mines with gentle agitation before making dilutions. duplicate standard points were prepared by serially diluting the standard stock solution (480pg/ml) 1:2 with standard diluent to produce 240pg/ml, 120pg/ml, 60pg/ml, and 30pg/ml solutions as illustrated in Figure 2-1. Any remaining solution should be frozen at -20°C and used within one month.

The dilution of standard solutions suggested is as follows: 20ml of wash buffer concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer.

| | | |
|---|---|---|
| 480pg/ml | Standard No.5 | 120µl Original Standard + 120µl Standard Diluent |
| 240pg/ml | Standard No.4 | 120µl Standard No.5 + 120µl Standard Diluent |
| 120pg/ml | Standard No.3 | 120µl Standard No.4 + 120µl Standard Diluent |
| 60pg/ml | Standard No.2 | 120µl Standard No.3 + 120µl Standard Diluent |
| 30pg/ml | Standard No.1 | 120µl Standard No.2 + 120µl Standard Diluent |




| Standard Concentration | Standard No.5 | Standard No.4 | Standard No.3 | Standard No.2 | Standard No.1 |
|---|---|---|---|---|---|
| 960pg/ml | 480pg/ml | 240pg/ml | 120pg/ml | 60pg/ml | 30pg/ml |

**Figure 2-1: The dilution procedure of standard solution**

**2-3-11-3 The Procedure:**

1. All reagents were prepared, standard solutions, and samples as instructed and brought to room temperature before use.
2. The strips were inserted in the frames for use. The unused strips are stored at 2-8°C.
3. A 50µl standard was added to the standard well.
4. 40µl of sample was added to sample wells and then 10µl of rat IL-10 antibody was added to sample wells, then 50µl of streptavidin-HRP was added to sample wells and standard wells. Mixed well and covered the plate with a sealer. Incubated for 60 minutes at 37°C.
5. The sealer was removed and the plate was washed five times with wash buffer. The wells were soaked with 300µl wash buffer for 30 seconds to 1 minute for each wash.
6. 50µl substrate solution A was added to each well and then 50µl substrate solution B was added to each well. The incubated plate was covered with a new sealer for 10 minutes at 37°C in the dark.

7. 50µl Stop Solution was added to each well, the blue color will change into yellow immediately.
8. The optical density (OD value) was determined for each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

The same procedure was done to measure the TNF-α level.

**2-3-11-4 Calculation of sample results:**

The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the samples by using a standard curve in Figures 2-2 and 2-3.

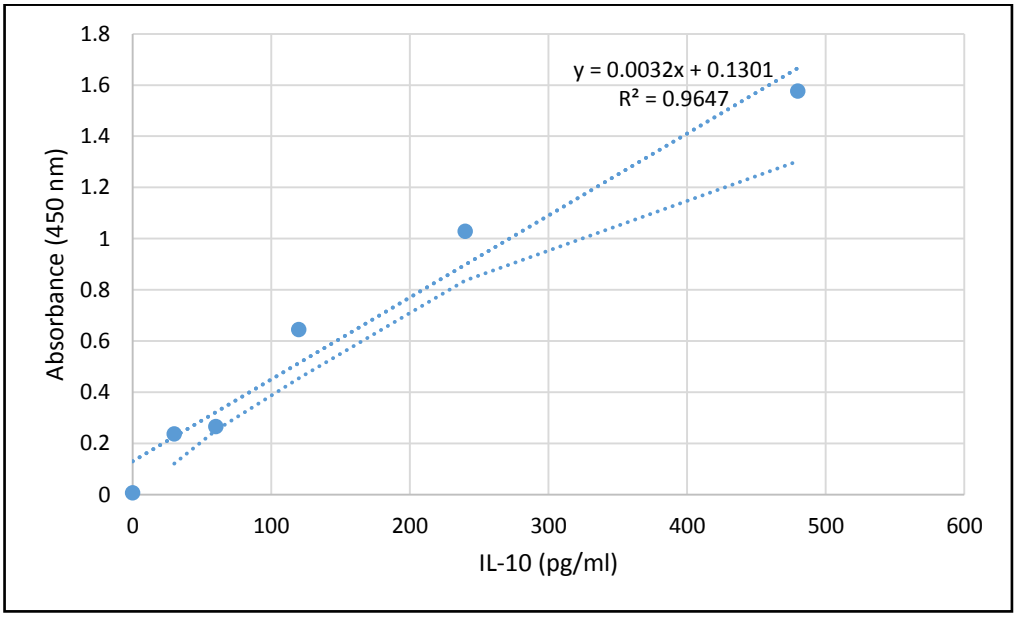



**Figure 2-2: Standard curve of IL-10**

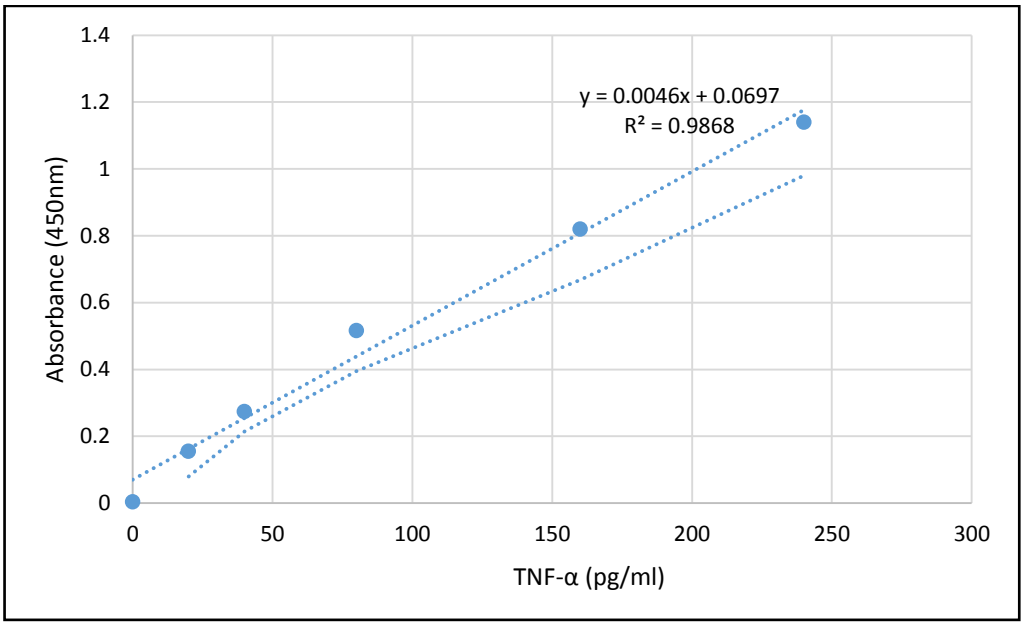



**Figure 2-3: Standard curve of TNF-α**

## 2-3-12 Studying the Histopathological Changes in Small and Large Intestines of Rats

The histological sections were done according to Bancroft (2019) in the following steps: -

**A) Fixation**

The tissue samples were fixed in a 10% neutral buffered formalin (NBF) for 24 hours to stop the bacterial activity immediately and stop the self-decomposition of the sample tissue.

**B) Dehydration**

The samples fixed with formalin solution were placed under tap water for 30 minutes, then passed in serial concentrations of ethanol (70, 80, 95,100, and 100% respectively) for 45min to each concentration to remove water and unbound fixative from the tissue without causing changes in the tissue composition or chemical content of the sample.

**C) Clearing**

The samples were cleared with xylene for three stages, 45 min to each stage to withdraw alcohol and make the sample more transparent.

**D) Infiltration**

After the clearing period, the sample was first placed with a mixture of xylene solution and molten paraffin wax at 58°C, in a ratio of 1:1, in an oven at 60°C for 15 minutes. Then the samples were embedded in paraffin wax at 60°C alone, for four stages, 30 min to each stage to ensure that the samples were infiltrated with the molten wax through it.

**E) Embedding and Making Blocks**

The molten wax used in the infiltration stage was poured into special molds prepared for this purpose. Pour the wax after embedding the sample prepared in the previous steps in it and pass a hot needle around the sample to remove air bubbles from the mold, then leave it until the wax hardens and store it in a cool place until the sample is cut.

**F) Trimming and Sectioning**

After the sample was hardened and separated from the mold, the wax molds containing the samples were trimmed with a sharp scalpel to remove excess wax from them, then they were placed on the base of the rotary microtome. The samples were cut serially, with a thickness of 5 micrometers, into cross-sections, these sections were held on glass slides using Myer's albumin and passed on a water path; they were dehydrated in an incubator at 45°C for 30 minutes.

**G) Staining**

The method was adopted to stain sample slides with hematoxylin (which is used to stain cell nuclei, elastic fibers, fibrin, supporting nerve cells, and muscle planning) and eosin (which dyes cytoplasm and muscle fibers in a dark pink color, while cartilage in a pale pink color) as follows:

1. The samples were placed in a xylene solution to remove the paraffin wax for 10 minutes and repeated twice for the same period.
2. A series of descending concentrations of ethyl alcohol was used, starting from 100%, 90%, 80%, and 70%, for two minutes for each concentration.
3. The samples were stained with hematoxylin solution for 5 minutes and then were washed to remove the excess dye under tap water for 5 minutes.
4. Eosin stain was used to stain the prepared sections for 3 seconds to stain the cytoplasm.
5. The sections were passed through a series of ascending concentrations of ethyl alcohol, 70%, 80%, 90%, and 100%, for two minutes for each concentration to dry them.
6. The sections were cleared with xylene twice for two minutes for each stage.

**H) Mounting**

Using Canada Balsam to attach the tissue sections, then covered them with a glass slide, making sure there were no air bubbles, then placed the sections on a hot plate to dry at a temperature of 37°C.

**I) Microscope Examination**

An optical microscope with different powers was used to suit the requirements of the current study to study the histological structure of the large and small intestines of the rats.

## 2-3-13 Statistical Analysis

Comparisons of prevalence data were tested using the Chi-square ($\chi 2$) test, ANOVA test, and Kruskal-Wallis tests with IBM SPSS Statistic (version 26).

# *Results & Discussion*

## 3-1 Specimens Collection and Sex Distribution of Inflammatory Bowel Disease (IBD) Patients

A total of 90 subjects were enrolled in this study; 60 IBD patients and 30 healthy subjects. IBD patients included 30 CD and 30 UC. Demographic and clinical data were collected from each patient. In addition, stool samples were collected from each subject and processed for isolation of *E. coli* and calprotectin test. Table 3-1 illustrated the sex distribution of IBD patients. The majority were females at 60% whereas the percentage of males was 40%.

**Table 3-1: Sex distribution of patients with IBD**

| **Sex** | **Type of Disease** | | **Total** |
|---|---|---|---|
| | **CD** | **UC** | |
| **Males** | 13 (21.67)% | 11 (18.33)% | 24 (40)% |
| **Females** | 17 (28.33)% | 19 (31.67)% | 36 (60)% |
| **Total** | 30 (50%) | 30(50%) | 60(100%) |
| **P-value** | 0.396 | | |

Sex distribution indicated that females were more susceptible to IBD than males. In line with these results, it was reported that sex differences in IBD have been identified for epidemiology, illness presentation, disease course, and complications (Greuter *et al*.,2020). There are two possible reasons for this result which are attributed to genetic susceptibility, hormonal differences, or both. Genetic susceptibility to IBD is linked to the X chromosome (Lungaro *et al*.,2023). Many loci on the X chromosome that are related to IBD such as *CD40LG- ARHGEF6* locus (Lee *et al*.,2017).

In an animal model, the loss of one allele of the X-linked gene that encodes for a chaperon, involved in the sex-specific risk of CD and UC was found only in female mice (Kudelka *et al*.,2016). Estrogens, on the other hand, hormone is known to influence the pathology and course of IBD ( Looijer-van Langen *et al*.,2011) There are Multiple studies, including a meta-analysis by Cornish *et al*. (2008) found that females who use oral contraceptives have a higher incidence of both CD and UC. Another study by Babakir-Mina, (2019) reported out of 102 UC female patients, 45 (44.1%) were more frequently under the use of contraceptive pills.

A similar trend was reported by Betterridge *et al* (2013) who indicated that the prevalence of IBD (UC and CD) was higher in females than males with increasing age, (417 versus 284 per 100,000). In a study from Denmark, where the incidence rates of both CD and UC in females were significantly higher than in males ( Lophaven *et al*.,2017). However, in contrast to these results, two studies conducted in Kurdistan of Iraq, in which IBD in males was more than in females (56.6% versus 43.4%) and (56% versus 45%) (Mohammed, & Amin,2022; Hamasur,2020), respectively. The reason for these results may be due to the different geographical areas in terms of the differences in diet, cultures, population density, and population distribution.

## 3-2 Age Distribution in IBD Patients

Table 3-2 showed the distribution of IBD subtypes according to age groups. From the table, all IBD patients below 15 years old were CD subtype (n=3). Moreover, most of the IBD patients below 25 years old had CD (8 out of 9). In contrast, most of the IBD patients over 55 years old had the UC subtype (12 out of 14). These results may be indicated that CD is the subtype of IBD that is prevalent among young patients, whereas UC is the subtype that is most prevalent among elderly patients. In other words, young ages were more susceptible to CD than older ages and vice versa. P-value <0.05 suggested that there is a statistically significant association between the type of disease and age distribution. In other words, the age distribution of patients with IBD appears to be significantly different.

**Table 3-2: Age distribution in IBD patients**

| Age groups | Type of Disease | | Total |
|---|---|---|---|
| | Crohn's patients | Ulcerative colitis patients | |
| **5-15** | 3(5%) | 0(0%) | 3(5%) |
| **16-25** | 5(8.33%) | 1(1.67%) | 6(10%) |
| **26-35** | 8(13.34%) | 4(6.66 %) | 12(20%) |
| **36-45** | 6(10%) | 5(8.33%) | 11(18.34%) |

| | | | |
|---|---|---|---|
| **46-55** | 6(10%) | 8(13.34%) | 14(23.33%) |
| **>55** | 2(3.33%) | 12(20%**)** | 14(23.33%) |
| **Total** | 30(50%) | 30(50%) | 60(100%) |
| **p-value** | **0.013** | | |

It was reported that CD tends to affect patients slightly earlier in life, at an average age of 15 to 25, whereas UC is more likely to set in between 25 and 35 years of age (Vatn MH &Sandvik,2015).

Genetic susceptibility and change in lifestyle with more dependence on fast food and lack of movement in young people and children may be among the important factors that affect the composition of the microbiome and lead to intestinal problems (Lakatos, 2009; Ng *et al*.,2017). Compared with younger IBD patients, genetics contribute less to the pathogenesis of older-onset IBD, with dysbiosis and dysregulation of the immune system playing a significant role in the development of IBD. Moreover, diagnosis may be difficult in older individuals; as multiple other common diseases can mimic IBD in this population. The clinical manifestations in older-onset IBD are distinct, and patients tend to have less of a disease course (Taleban *et al*.,2015). These results came in accordance with a study from 9 European countries which showed that CD hospitalization was age-distributed, with a large peak in younger patients followed by a small peak in older patients while a small peak in younger patients was followed by a large peak in older patients for UC hospitalization (Sonnenberg,2010).

Another study from Iraq reported that the mean age for UC patients was 46.36 years old in the elderly stage (> 45 years of age) making the highest proportion of UC patients ( Babakir-Mina, 2019). Similar age distribution has been reported in Asia, where the median age of diagnosis in patients with CD and UC was 34 and 42 years, respectively (Ng *et al*.,2016).

## 3-3 Symptoms of Patients with IBD

Figure 3-1 illustrated the prevalence of symptoms in IBD patients. Diarrhea was the most common symptom in both CD and UC patients with 40% and 46% respectively, whereas in 30% of UC patients, there was blood in the stool more frequently than in CD patients. Furthermore, constipation was reported in CD patients at 10% slightly higher than in UC patients at 7%. In addition, nausea was more reportedly seen in CD patients 30% as compared to UC patients 17%. The symptoms of inflammatory bowel disease vary depending on the severity of the inflammation and the part of the intestine that is involved.

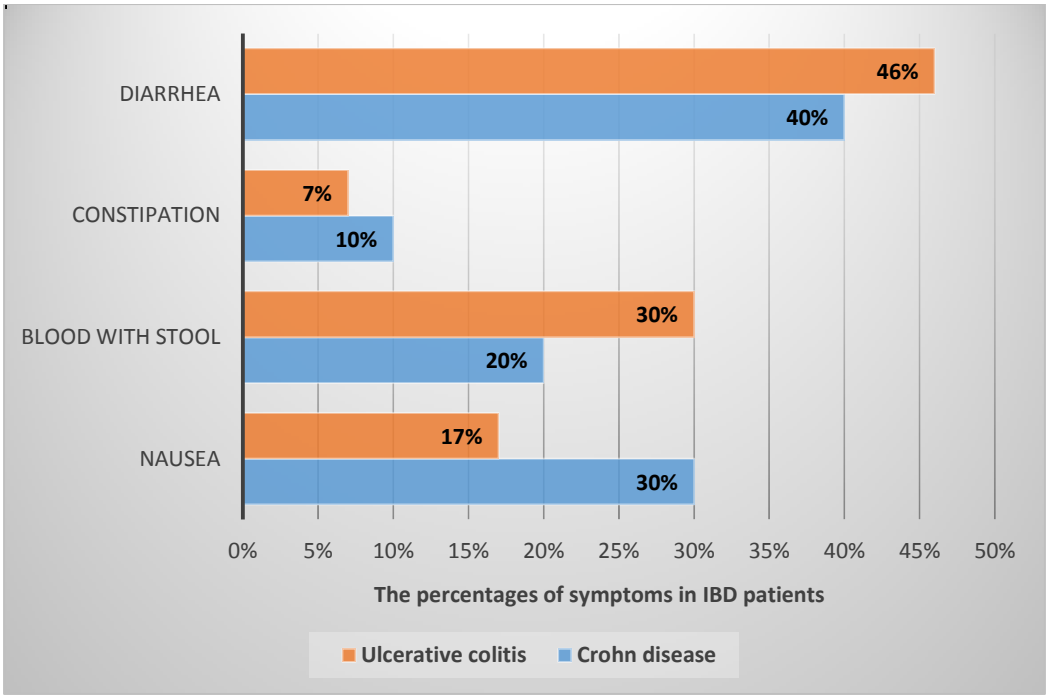



**Figure 3-1: Distribution of symptoms in IBD patients**

The severity of the symptoms can range from mild to severe, periods of active illness are followed by periods of remission. Diarrhea, fatigue, abdominal pain and cramping, blood in stool, reduced appetite, and unintended weight loss are all signs and symptoms of CD and UC ( Chang & John,2020).

In the current study, diarrhea with or without blood was the most common symptom among IBD patients. The pathogenesis of IBD-associated diarrhea is multifactorial, with mucosal damage caused by persistent inflammation leading to dysregulated intestinal ion transport, impaired epithelial barrier function, and increased pathogen accessibility to the intestinal mucosa. The primary cause of electrolyte retention and water accumulation in the intestinal lumen in IBD is altered expression and/or function of epithelial ion transporters and channels (Anbazhagan *et al.*,2018). Constipation may occur in IBD patients for many reasons which include medications such as antidiarrheal drugs, iron supplements, calcium channel blockers, and certain pain relievers or low fiber diets, or because of what is called "strictures" that may happen to CD patients where the intestines become narrow due to severe inflammation and that can block or slow the passage of stool through the bowels (Cynthya,2019). A survey by Aldakhil (2022) study reported that diarrhea and blood with stool in UC patients were the most common symptoms. Blood with stool occurs more frequently with UC (83%) compared to CD (40%) (Yangyang & Rodriguez, 2017). A study that included 233 CD and 150 UC patients, revealed the most common symptoms in CD were fatigue and abdominal pain while passage of blood with bowel movements and loose/watery stool were most common in UC (Perler *et al.*,2019).

However, the differential diagnosis between CD and UC patients depending on clinical symptoms alone is difficult because the two subtypes are similar in most symptoms.

Therefore, clinicians should also consider besides symptoms and laboratory tests, pathological, radiological, and endoscopic findings (Lee & Lee, 2016).

## 3-4 The Extraintestinal Manifestations (EIMs)

Figure 3-2 showed the important and frequent extraintestinal manifestation that is reported in IBD patients. Arthritis was the most common extraintestinal manifestation with 60% in UC and 58% in CD patients. The second common manifestation was eye disorder which was reported in 17% of UC and 11% of CD patients. Whereas, skin manifestation and formation of fistula were third with a high percentage of CD in both disorders.

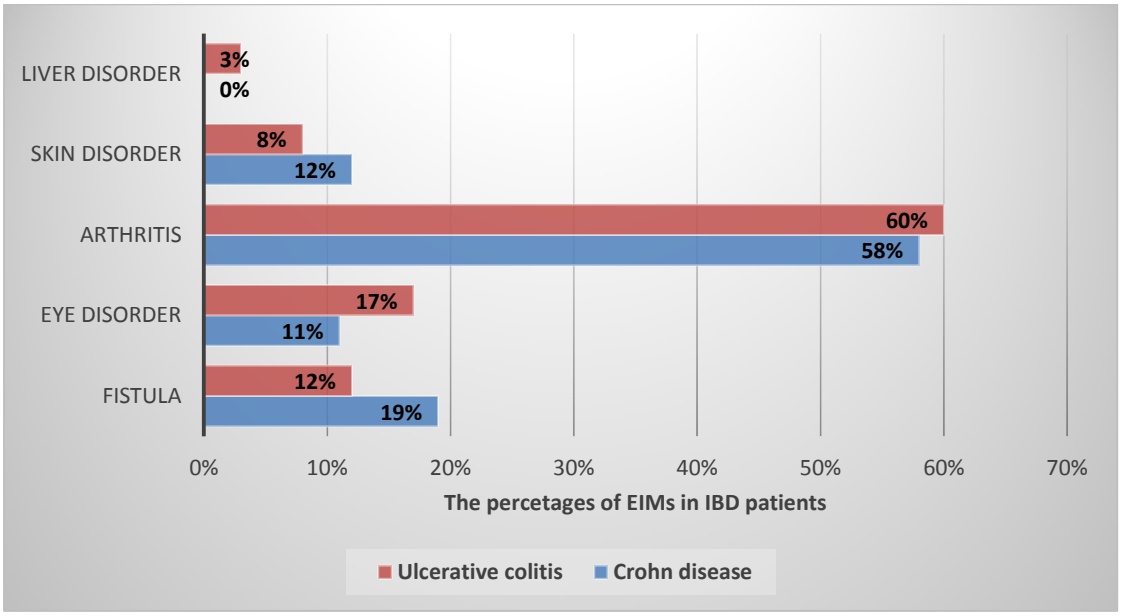



**Figure 3-2: Distribution of extraintestinal symptoms in IBD patients**

Extraintestinal manifestation most frequently affects joints, skin, and eyes, and can also less frequently involve other organs such as the liver, lungs, and pancreas (Vavricka *et al.*,2015). According to a Swiss IBD cohort research, 25% of individuals with IBD had several EIMs, (in some cases up to five separate EIMs) and these disorders can arise before the diagnosis of IBD in 25.8% of cases (Vavricka *et al.*,2011). There are many mechanisms by which gut microbiota drive the pathogenesis of EIMs such as the translocation of luminal bacteria across a leaky intestinal barrier which disrupts immune responses at extraintestinal locations due to common epitopes, or by soluble microbial-derived factors, e.g. Lipopolysaccharide (LPS), may be released into the circulation and promote inflammation at extraintestinal sites, or through the microbiota-derived metabolites, e.g. from the metabolism of bile acids or the generation of short-chain fatty acids, both of which could alter immune signaling (Hedin *et al.*,2019).

A study by Mendoza *et al.*, (2005) which came consistent with this study indicated that the most common EIMs is arthritis which was 51.5% in UC and 42.2% in CD. EIMs have a

direct relationship with the activity of bowel diseases, the most common types include pauciarticular arthritis, oral aphthous ulcers, erythema nodosum, and episcleritis (Trikudanathan *et al*., 2012). Cutaneous disorders may precede or occur at the same time, or follow a diagnosis of IBD; they occur in 22–75 % of CD and 5–11 % of UC patients. The visual system, in another hand, is one of the most frequently affected in IBD patients, and Crohn himself described the first case of ocular involvement in IBD patients (Marotto *et al*.,2020). The prevalence of ocular manifestations varies from 0.3 % to 13 % in IBD patients, and it is more frequent in CD patients than in UC patients (Marotto *et al*.,2020). Fistulae represent an important complication in patients suffering from IBD and about one-third of CD patients suffer from fistulae formation. Fistulae originate from an epithelial defect due to destructive inflammation (Scharl & Rogler, 2014). Tan *et al*., (2023) showed that fistulas are common in CD and less in UC in which this result is similar to this study.

It is important to differentiate between extraintestinal manifestations and complications of IBD, the latest ones include iron-deficiency anemia and osteoporosis are consequences of disease-specific treatment and intestinal obstruction. However, extraintestinal manifestations and complications strongly influence the quality of life of IBD patients (Ott & Schölmerich, 2013).

## 3-5 Fecal Calprotectin(FC) Detection:

In this study, the FC test was used to determine the IBD patients with active inflammation. Fecal calprotectin results were illustrated in Table 3-3, FC levels in 48 patients were in the range between 50-200 (µg/mg) which is interpreted as having active gastrointestinal inflammation. Whereas the level of FC in 12 patients was more than 200(µg/mg) which was interpreted as having acute inflammation during the time of obtaining stool samples. These results indicated that all 60 patients were in a relapsing disease period.

**Table 3-3: Fecal calprotectin results**

| **Type of Disease** | **Concentrations of FC tests** | | **Total** |
|---|---|---|---|
| | **50-200 (µg/mg)** | **>200(µg/mg)** | |
| **CD** | 23(38.33)% | 7(11.67)% | 30(50%) |
| **UC** | 25(41.67)% | 5(8.33)% | 30(50%) |
| **total** | 48(80)% | 12(20)% | 60(100%) |
| **p-value** | 0.374 | | |

In the intestinal tract, inflammatory diseases with different etiology cause increased mucosal permeability that induces migration of granulocytes and monocytes into the intestinal lumen. The activation and death of these cells release a great amount

of calprotectin, which is excreted in the feces in pathological conditions, both inflammatory and neoplastic, Crohn's disease, ulcerative colitis, cystic fibrosis, rheumatoid arthritis, bacterial infections, and gastric cancer (Roca *et al*.,2017). There are many factors influencing the results of FC such as type of diet, type of used drug, age, and microbiome profile (Koninckx *et al.*,2021) in healthy individuals FC has been shown to increase at the extremes of age >65 and in infants <1 year. The reasons for the observed increase in the elderly include alterations in diet and lifestyle, specific age-related changes in inflammatory cells in the gastrointestinal mucosa, and changes in both cellular and humoral immunity (Ayling & Kok,2018). Whereas, at early ages the digestive tract undergoes development and the immunological barrier's first line of defense, which binds to antigenic substances, does not completely mature (Anderson *et al*.,2012). FC is important for predicting imminent clinical relapse and also has a role in predicting mucosal healing (Bjarnason, 2017; Yamamoto *et al*.,2014). If FC level below 60 (µg/mg) accurately predicted the depth of remission. Moreover, FC can be employed instead of colonoscopy in a treat-to-target paradigm in UC patients (Patel *et al*.,2017). In a study by Gisbert *et al*., (2009), was revealed that calprotectin concentrations in patients who suffered a relapse were higher than in non-relapsing patients.

**3-5-1 Sex Distribution in the FC Test**

Sex distribution in the FC test is illustrated in Table 3-4. Although there were no significant differences between sexes in IBD subtypes and this test in both concentrations (FC >200 µg/mg and FC<200 µg/mg) P-value>0.05. The percentage of males was 40% while females 60% with a high rate in the concentration of less than 200 µg/g in both sexes. These results indicated that females had high levels of fecal calprotectin and this is because the number of infected women with IBD was more than males. A study by Mindemark & Larsson, (2012) showed the concentration of FC test between 50-100 µg/g was slightly higher in females than males (68.5% versus 65.5%). In another study which came opposite to this study, the FC concentration in males was more than in females ( Amati *et al*.,2006). That may be attributed to the type of diet and the drugs used by patients such as nonsteroidal anti-inflammatory drugs which raise the level of calprotectin in patients (Ayling& Kok, 2018).

**Table 3-4 Sex distribution in the FC test**

| Sex | Concentrations of FC test | | Total |
|---|---|---|---|
| | FC>200 µg/mg | FC<200 µg/mg | |
| males | 5(8.33%) | 19(31.67%) | 24(40%) |
| females | 7(11.67%) | 29(48.33%) | 36(60%) |
| total | 12(20%) | 48(80%) | 60(100%) |
| p-value | 0.573 | | |

## 3-6 Isolation and Identification of *Escherichia coli*

From all the stool samples obtained from the subjects (patients and healthy), 90 *E. coli* isolates were isolated and identified by using standard microbial methods according to Bergey's Manual of Systematic Bacteriology, 2nd edition (Brenner *et al*., 2005). The identification was confirmed by PCR technique.

### 3-6-1 Cultural characteristics

On MacConkey agar, which is a selective and differential medium, the colonies of *E. coli* appeared as round, small, pink, and lactose fermenters. Lactose fermented isolates were cultured on Eosin Methylene Blue (EMB) agar. Positive colonies appeared as a green metallic sheen with a dark center; this is due to rapid lactose fermentation which produces acids, in turn, lower the pH of the medium that encourage EMB dye absorption by bacterial colonies, and lead to the production of green metallic sheen (Bachoon & Wendy, 2008).

### 3-6-2 Microscopic characteristics

The isolates appeared as Gram-negative coccobacilli or bacilli shaped appearance and arranged in single or in pairs were assumed to be *E. coli* under a light microscope.

### 3-6-3 Biochemical tests

As is shown in Table 3-5 all isolates were negative for to oxidase test and positive for catalase. Kligler iron agar (KIA) developed an acidic slant and bottom, $H_2S$ production was negative with gas production (A/A + -), KIA test indicated that these isolates were capable of

fermenting both glucose and lactose sugars which create acidic byproducts that led to turning the phenol red indicator in the media to yellow, in addition, gas production as a result of fermentation processes, but the inability to produce $H_2S$ was investigated (Patricia,2021).

**Table 3-5: Biochemical test results for *Escherichia coli***

| Biochemical test | Result |
|---|---|
| **Catalase production** | **+** |
| **Oxidase production** | **-** |
| **Kliglar iron agar (KIA)** | **A/A + -** |
| **Lactose fermentation** | **+** |

## 3-7 Molecular Confirmation of *E. coli* Isolates and Detection of Toxins Genes

In this study, polymerase chain reaction (PCR) was used to confirm the microbiological identification of *E. coli* isolation. Traditional methods of detection, though reliable and efficient, require several days to weeks before results are obtained. Furthermore, phenotypic properties by which the bacteria are identified may not always be expressed. The introduction of PCR in microbial diagnostics has been established in research laboratories as a valuable alternative to traditional detection methods. Speed, a good detection limit, selectivity, and specificity are among its most important advantages (Malorny *et al.*,2003).

### 3-7-1 DNA Extraction

DNA was extracted and purified from *E. coli* isolates using a commercial purification kit. Results showed that the purity of extracted DNA ranged between 1.7 and 1.9. The optical density ratio $OD_{260/280}$ was considered well in the expected range of 1.7 to 2 (Deng *et al.*, 2001).

### 3-7-2 Confirmative of *E. coli* Gene:

All *E. coli* isolates were confirmed by PCR technique by the amplification of *the 16S rRNA* gene, with product size 100bp Figure 3-3. *E. coli* is the most common and one of the first bacteria to colonize the digestive tract of newborns after birth which is characterized by

great versatility and metabolic flexibility that allows its survival in different niches (Sina *et al*.,2022).

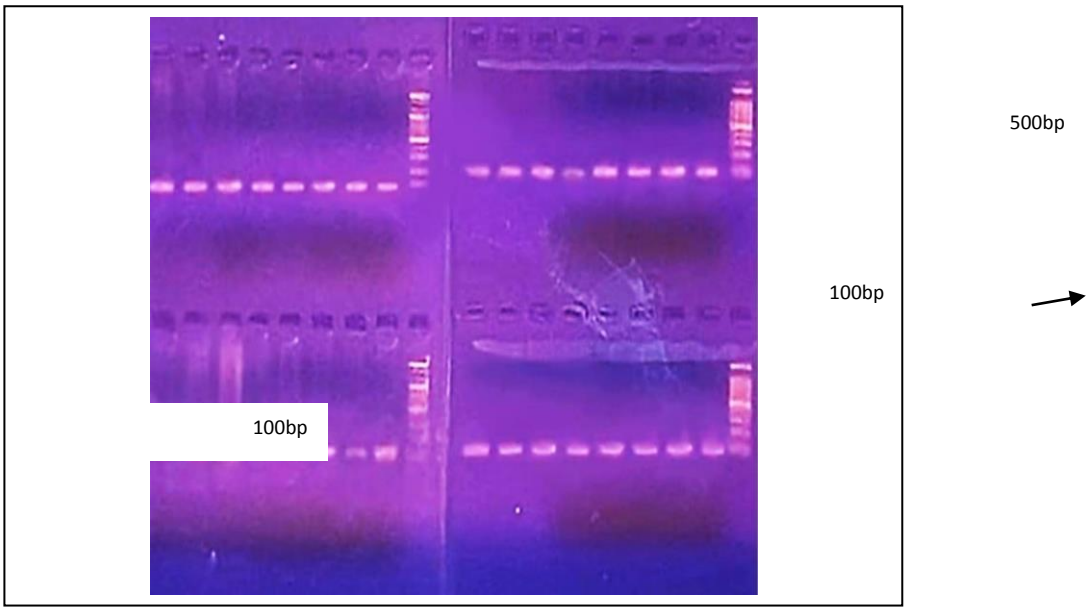



**Figure 3-3: Electrophoresis of *16S rRNA* PCR of amplified gene product (size100 bp), DNA template extracted from *E. coli* isolates. Electrophoresis was carried out using 1% agarose gel and stained with ethidium bromide at 70 volts for 1-2 hrs. All lanes showed the presence of the single band. DNA Ladder (from 100 to 1200 bp) was used.**

16S and 23S rRNAs form the structural core of the ribosome, they are believed to be least likely to experience horizontal gene transfer between species. Based on the species-specific nature of rRNA and their omnipresence in all bacteria, the rRNA genes, especially those for 16S rRNA, have long been used for the specific identification of bacterial species ( Miyazaki *et al*., 2017). In addition, *E. coli* is considered a member of the intestinal microbiome of over 90% of individuals (Martinson & Walk, 2020). Whereas the abundance of *E. coli* is 3–4 logs higher in patients with IBD (Kotlowski *et al*.,2007).

### 3-7-3 PCR–Based Toxin Gene Detection of Colibactin Gene

Figures 3-4,3-5 illustrated the amplification of colibactin gene *clbB* with a product size of 280bp by using a DNA template extracted from *E. coli* isolates isolated from CD, UC, and control stool.

The PCR amplification results in this study showed that 12 out of 90 *E. coli* isolates (60 of the IBD patients and 30 of the control) had this gene 4 isolates from CD and 6 from UC whereas 2 isolates from the control Figure 3-6.

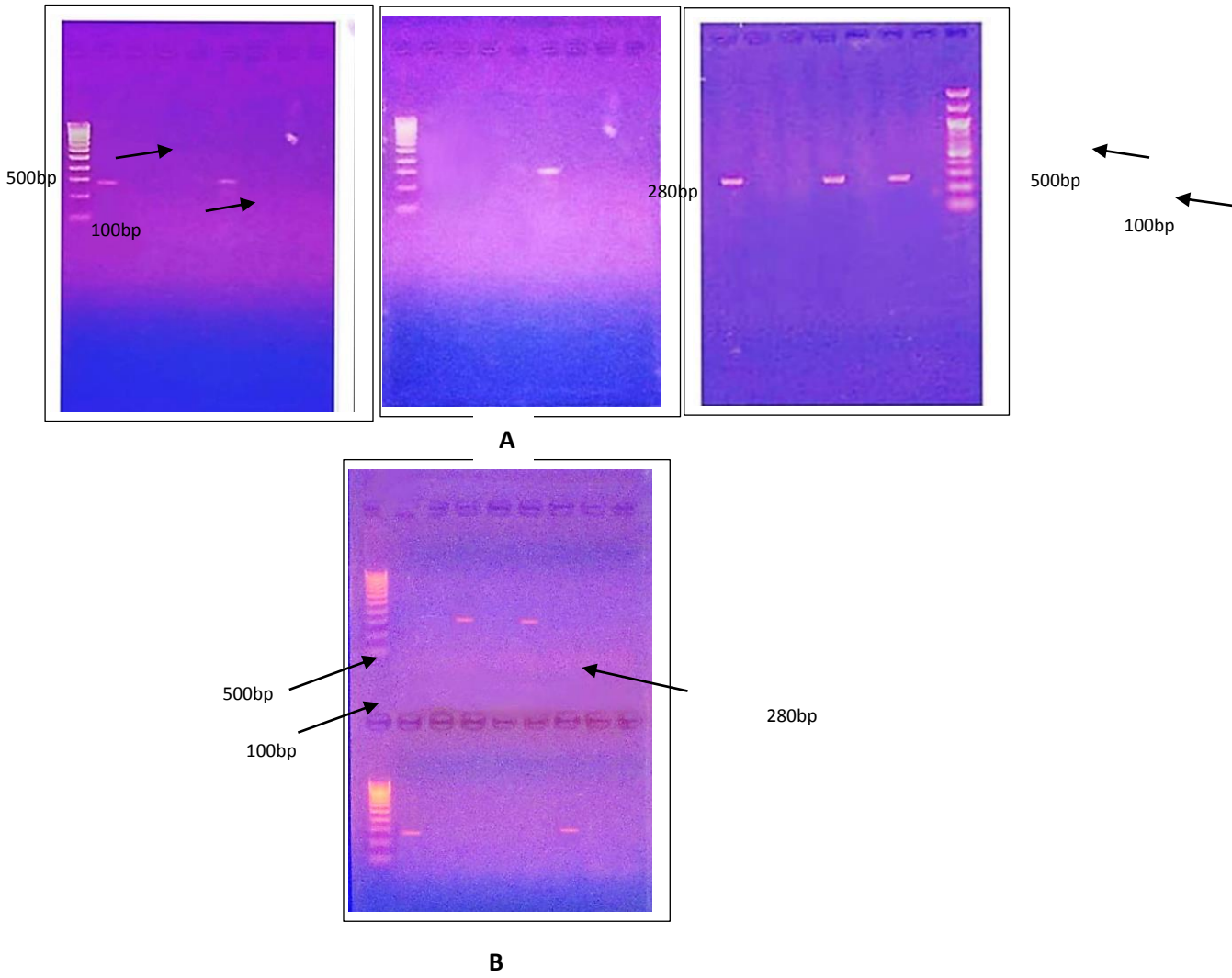



**Figure 3-4: Electrophoresis of *clbB* PCR of amplified gene product (size 280 bp), DNA template extracted from *E. coli* isolates from UC patients (A) and CD patients (B). Electrophoresis was carried out using 1% agarose gel and stained by ethidium bromide at 70 volts for 1-2 hrs. DNA Ladder (from 100 to 1200 bp) was used.**

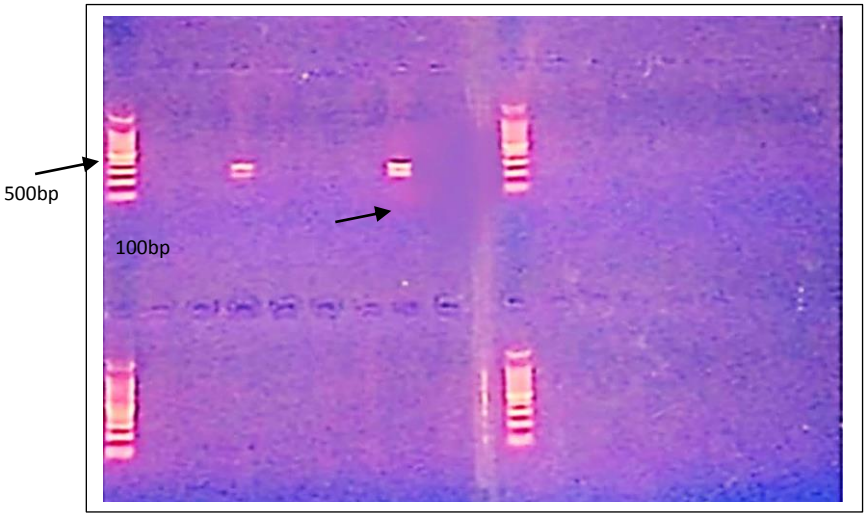



**Figure 3-5: Electrophoresis of *clbB* PCR of amplified gene product (size 280 bp), DNA template extracted from *E. coli* isolates from control. Electrophoresis was done using 1% agarose gel and stained with ethidium bromide at 70 volts for 1-2 hrs. Lanes showed the presence of a single band. DNA Ladder (from 100 to 1200 bp) was used.**

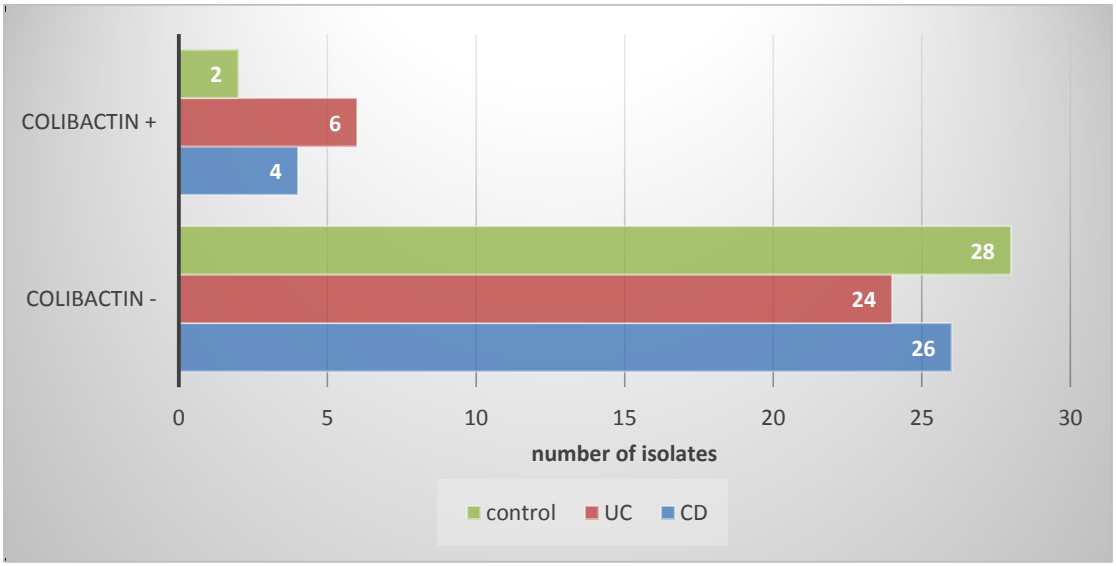



**Figure 3-6: Distributions of toxin gene colibactin in the isolates of CD, UC, and control**

The prevalence of the colibactin gene in IBD patients' stool isolates according to this study was low. The reason for these results may be attributed to the type of specimens, in this study *E. coli* was isolated from the patient's stool, whereas in other studies such as in Arthur *et al*., (2012) study *E. coli* were isolated from mucosal biopsy of IBD patients,

reported a high rate of the colibactin-encoding gene in these isolates about 40% and a study by Prorok-Hamon *et al*.,(2014) showed that the rate was 34% mucosal biopsy also used. Pathogenic *E. coli* which harbors the colibactin gene are frequently abundant in mucosal tissue taken from patients with colorectal cancer (CRC) and inflammatory bowel disease (Dougherty & Jobin, 2021). Evidence suggests that *E. coli* generating colibactin in IBD patients induces epithelial oxygenation, reduces mucosal barrier integrity, and promotes bacterial biofilm formation. (Tang-Fichaux *et al*.,2021). These isolates with the colibactin gene were highly persistent in the gut, and induced colon inflammation, epithelial damage, and cell proliferation (Raisch *et al.,*2014).

The results of this study were close to Dubinsky *et al*., (2020) study which reported that the prevalence of the colibactin gene in *E. coli* isolates from fecal samples ranged from 20.9% to 22% in both IBD phenotypes. Furthermore, patients with IBD, such as Crohn's disease and ulcerative colitis, who were exposed to high levels of colibactin exposure, and years of chronic inflammation have a higher lifetime risk of developing colorectal cancer (CRC) (Clarke &Feuerstein, 2019) (Nougayrède *et al*.,2006). In colitis-susceptible interleukin-10-deficient (*Il10*$^{-/-}$) mice colonization with the *E. coli* with *(pks)* genotoxic island which produces colibactin promoted invasive carcinoma, and the deletion of this island from *E. coli* decreased tumor multiplicity and invasion ( Arthur *et al*.,2012).

**3-7-4 PCR–Based Toxin Gene Detection of Microcin B17 Gene:**

The PCR amplification of the toxin microcin B17 ( *McbA*) gene with a product size of 203bp for all *E. coli* isolates showed high prevalence in 27 isolates from CD and 25 isolates from UC, whereas 5 *E. coli* isolates only were found to harbor ( *McbA*) gene from control. , Figures 3-7, 3-8, and 3-9.

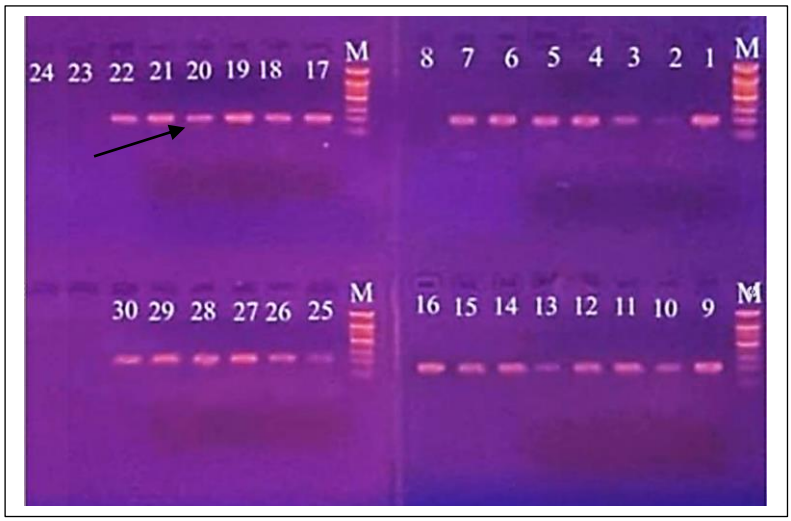


**Figure 3-7: Electrophoresis of *McbA* PCR of amplified gene product (size 203 bp), DNA template extracted from *E. coli* isolates from Crohn's disease. Electrophoresis was carried out using 1% agarose gel and stained with ethidium bromide at 70 volts for 1-2 hrs. DNA Ladder (from 100 to 1200 bp) was used.**

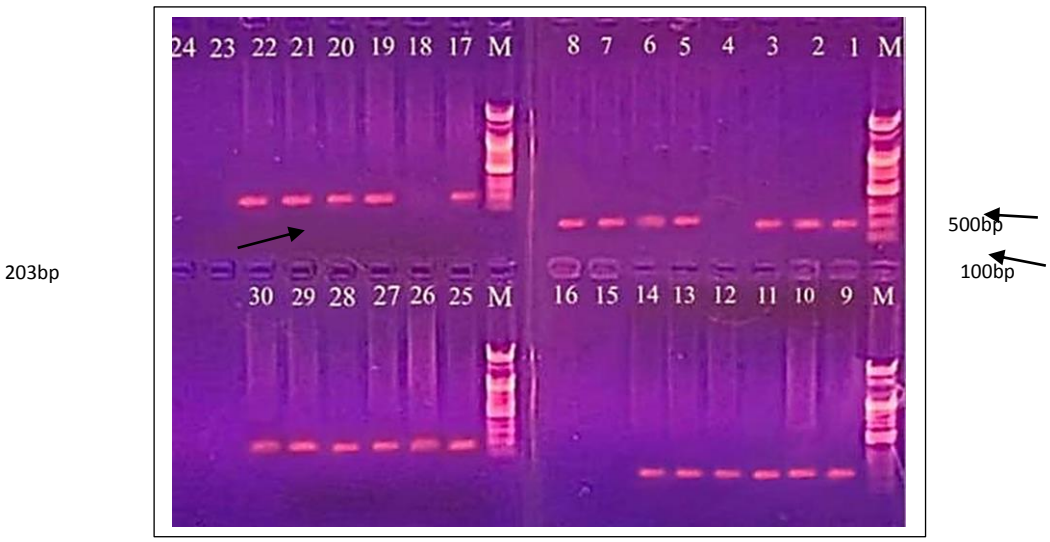



**Figure 3-8: Electrophoresis of *McbA* PCR of amplified gene product (size 203 bp), DNA template extracted from *E. coli* isolates from ulcerative colitis. Electrophoresis was carried out using 1% agarose gel and stained with ethidium bromide at 70 volts for 1-2 hrs. DNA Ladder (from 100 to 1200 bp) was used.**

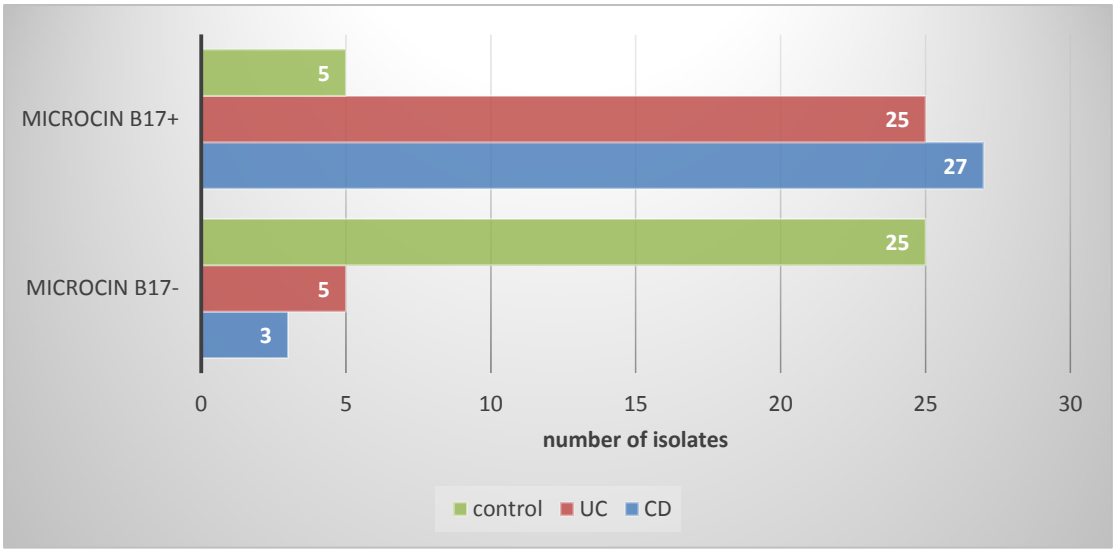



**Figure 3-9: Distributions of microcinB17 gene in *E. coli* isolates of CD, UC, and control**

*E. coli* isolates from IBD patients possessed the microcinB17 gene with a higher percentage than the other bacterial toxin gene (colibactin). Microcins are low molecular mass bacteriocin, produced by *E. coli*, under stress conditions, which respond very well to changes in pH, protease activity, or temperature alterations as a mechanism for competition and bacterial fitness (Markovic *et al*.,2022) by activation of the bacterial SOS response (Mosso *et al*.,2019). Many studies have shown that one of the reasons that lead to pathologic changes (dysbiosis) in the GI of IBD is the production of microcins including microcinB17 (Hansen & Sartor,2015).

In a study by Iyer, *et.al* (2018) they defined microcinB17 as an aromatic organic compound (oxazole class) which is considered a source of microbial gastrointestinal inflammation inducers, that cause CD1d-dependent intestinal inflammation and modulates natural killer T cell-dependent inflammation (Collin *et al*.,2013).

The findings of this study were in agreement with Micenková *et al* (2018) study which reported that the frequency of bacteriocinogenic isolates was significantly higher in IBD *E. coli* (70%) compared to fecal commensal *E. coli* isolates, including a higher prevalence of the microcin B determinant.

**3-7-5 The Correlation Between Colibactin and Microcin B17 Genes in Isolated Bacteria**

Tables 3-6 and 3-7 showed the prevalence of toxins genes (colibactin and MicrocinB17) in isolated bacteria from CD and UC patients. The isolates that possess the two genes together are 4 isolates in CD and 6 isolates in UC. While the isolates that have the microcin B17 gene only 23,19 isolates in both CD and UC sequentially, and the isolates that do not have any of the toxin genes are 3 in CD and 5 in UC, whereas there are no isolates that have colibactin gene only. In conclusion, all isolates that have colibactin own microcin genes but the opposite is not.

**able 3-6: The prevalence of toxin genes in CD isolates**

| **Toxins genes** | | **Microcin B17** | | **total** |
|---|---|---|---|---|
| | | **+** | **-** | |
| **Colibactin** | **+** | 4(13.33%) | 0(0.00%) | 4(13.33%) |
| | **-** | 23(76.67 %) | 3(10%) | 26(86.67%) |
| **Total** | | 27(90%) | 3(10%) | 30(100%) |

**Table 3-7: The prevalence of toxin genes in UC isolates**

| Toxin genes | | Microcin B17 | | Total |
|---|---|---|---|---|
| | | + | - | |
| Colibactin | + | 6(20%) | 0(0.00%) | 6(20%) |
| | - | 19(63.33%) | 5(16.67%) | 24(80%) |
| Total | | 25(83.33%) | 5 (16.67%) | 30(100%) |

There are many studies where *E. coli* isolates harbored microcins and colibactin genes together, in a study by Massip *et al*.,2019 demonstrated that *Escherichia coli* Nissle 1917 which has been used as a probiotic for over a century, produces the genotoxin colibactin and microcins, these isolates has been linked to the virulence of certain *E. coli* strains and could promote colorectal cancer. Another study where uropathogenic *E. coli* (UPEC) had a functional synergy demonstrated between microcin, salmochelin, and colibactin islands that would promote urinary tract colonization, and these isolates were more virulent than other UPEC ( Massip *et al*.,2020).

## 3-8 Pathogenicity *in vivo* Study of *E. coli* Isolates From (CD and UC) Patients in Rats Gastrointestinal System

To our knowledge limited data are available about the pathogenic roles of microcin B17 in natural hosts or experimental animal models. Such information is crucial for elucidating pathogen–host interactions. Furthermore, observing serological changes in the sera as well as histological changes in the intestine of infected rats is important to demonstrate the role of the toxin.

This study aimed to show whether *E. coli* with microcin B 17gene (*E. coli*/+ *McbA*)isolated from IBD patients could induce inflammation and changes in the intestines of experimental animal models.

Female rats were used in this study, the rats were divided into five groups: the first group, the control group was orally given normal saline, the second group was orally given *E. coli*/+ *McbA* isolated from the stool of UC, the third group rats were orally given *E. coli*/+*McbA* isolated from the stool of CD patients, fourth group rats were orally given *E. coli* do not harbor microcin B17 gene (*E. coli*/- *McbA*) isolated from the stool of UC patients and the last group rats were orally given *E. coli* do not harbor microcin B17 gene (*E. coli*/- *McbA*) isolated from the stool of CD patients.

The following criteria were used in the selection of isolates to be enrolled in this study:

1. All rats were tested before the beginning of the experiment for the presence of the microcin B17 gene in their stool by using the PCR technique. The results were negative.
2. For the group of rats that were orally given *E. coli* non-producing microcin B17 (*E. coli*/-*McbA*). The isolates were selected from the bacteria that do not have either the microcin B17 gene or colibactin (Table 3-6,3-7).
3. For the group that was orally given *E. coli*/+ *McbA* isolated from Crohn's patient's stool, this isolate was selected from the bacteria that harbor microcin B17 gene only and negative for colibactin gene, the same thing for the group that was orally given *E. coli*/+ *McbA* isolated from patients stool of UC (Tables 3-6,3-7).

## 3-8-1 Cytokines Levels in the Blood of the Rats After Completion of Dosage

Cytokines play a pivotal role in the regulation of mucosal inflammation by promoting leukocyte migration to sites of inflammation ultimately leading to tissue damage and destruction. In recent years, experimental studies in rodents have led to a better understanding of the role played by these inflammatory mediators in the development and progression of colitis (Singh *et al*.,2016). The imbalance between proinflammatory and anti-inflammatory cytokines that occurs in IBD results in disease progression and tissue damage and limits the resolution of inflammation (Guan & Zhang, 2017). It is a reported increase in the inflammatory cytokines IL-16, IFN-γ, IL-1β, TNF-α, and IL-10 in IBD patients when compared to healthy donors (Singh *et al*.,2016; Meng *et al*.,2019).

#### 3-8-1-1 Serum Interleukin-10 (IL-10) Levels in Rats Serum

Table 3-8 illustrated the results of the IL-10 levels in the serum of rats groups enrolled in this experiment. The highest level of interleukin was detected in the sera of group II rats at 110.91 (pg/ mL) which was dosed (*E. coli*/+ *McbA*) isolated from UC patients compared to control group I with 94.16 (pg/ mL). There were no significant differences remarked between groups with p-values>0.05.

**Table3-8: Comparison among under-studied groups in IL-10 levels in Serum**

| Groups | Mean pg/mL | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| V | 99.15 | 17.32 | 7.75 | 0.118 |
| IV | 103.15 | 13.01 | 5.82 | |
| III | 102.87 | 6.29 | 2.81 | |
| II | 110.91 | 3.86 | 1.73 | |
| I | 94.16 | 9.89 | 4.42 | |

Kruskal-Wallis tests have been utilized to discern statistically significant differences across multiple independent groups.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

**I: control, II: rats dosed *E. coli/+ McbA* isolates from UC, III: rats dosed *E. coli/*$^{+}$ *McbA* isolates from CD, IV: rats dosed *E. coli/- McbA* isolate from UC, V: rats dosed *E. coli/*$^{+}$ *McbA* isolates from CD**

These results indicated that isolated bacteria from IBD patients' stool caused immune responses and stimulated the production of cytokines such as IL-10 in the experimental animals. Anti-inflammatory IL-10 is a key immunoregulatory cytokine that suppresses and terminates inflammatory immunological responses, primarily by inhibiting monocyte and macrophage activity and downregulating the production of Th1-derived cytokines. The relative deficiency of IL-10 in patients with ulcerative colitis may contribute to persistent inflammatory changes (Li & He, 2004; Saxton *et al*.,2021). IL-10 inhibits immune responses and reduces the damage caused by inflammation (Bucker *et al*.,2014). Schmitz *et al*.( 2019) developed colitis in IL-10 deletion gene mice, when they infected them with adherent and invasive *Escherichia coli* (AIEC), they developed chronic inflammation in the small and large intestines. In another study IL-10-deficient mice (IL-10(-/-)) spontaneously developed intestinal inflammation characterized by discontinuous transmural lesions affecting the small and large intestines, dysregulated production of proinflammatory cytokines, indicating that endogenous IL-10 is a central regulator of the mucosal immune response (Li & He, 2004).

A meta-analysis study illustrated that the serum IL-10 level increased in UC patients unlike in the control group, and it is contributing to the pathogenesis and progression of disease in these patients (Meng *et al*.,2019). Loss-of-function of IL-10 by mutations in IL-10 or IL-10 receptor (IL10R) genes in some people, developed severe, medical-refractory, infantile-onset IBD (Werner *et al*.,2020; Iyer & Cheng, 2012). A study by Tomoyose *et al.*,(1998) indicated that after the induction of colitis in mice, there was a time-dependent increase in tissue TNF-α level, followed by a peak of the IL-10 levels. *E. coli* was a stronger inducer of IL-10 in animal models with colitis. However, massive production of IL-10 at the early stages of acute inflammation may result in the inhibition of TNF-α and may be responsible for the ineffective elimination of infectious agents (Strus *et al*.,2015). The results were consistent with a study by Abdul-Zahraa *et al*., (2019) which reported that the administration of microcin to mice, elevated serum levels of TNF-α to 21.86 (pg/ml) when compared to the control 14.32(pg/ml) and IL-10 to 27.24 (pg/ml) compared to control

19.54(pg/ml). The levels of IL-10 may decrease in patients with IBD with chronic inflammation and persistent inflammatory changes (Ishizuka *et al*.,2001).

**3-8-1-2 Rat Tumor Necrosis Factorα (TNF-α)**

In Table 3-9, TNF-α levels recorded significant differences among all studied groups. The means were (21.16, 14.23, 15.63,13.59) pg/mL in groups II, III, IV, and V respectively (P≤0.01) group II had the highest levels and revealed differences with groups III, IV, and group V. Appendix4

**Table 3-9: Comparison among under-studied rat groups of serum TNF-α levels**

| Groups | Mean pg/mL | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| V | 13.59 | 2.31 | 1.03 | 0.0001** |
| IV | 15.63 | 2.30 | 1.03 | |
| III | 14.23 | 3.44 | 1.54 | |
| II | 21.16 | 2.75 | 1.23 | |
| I | 11.83 | 1.97 | 0.88 | |

ANOVA tests have been utilized to discern statistically significant differences across multiple independent groups.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

**I: control, II: rats dosed *E. coli/+ McbA* isolates from UC, III: rats dosed *E. coli/+ McbA* isolates from CD, IV: rats dosed *E. coli/- McbA* isolates from UC, V: rats dosed *E. coli/- McbA* isolates from CD**

Pro-inflammatory cytokine TNF-α has a role in the development of intestinal inflammation and is important in the pathophysiology of IBD and tumor formation (Ruder *et al*.,2019).

Increased levels of the proinflammatory cytokine TNF-α in the gut are linked to disease activity and severity. Levels of TNF-α are significantly increased in response to intestinal inflammation. Indeed, clinical studies have demonstrated that serum TNF-α levels are elevated in patients with IBD (Leppkes & Neurath,2020). An increase in TNF-α induces cell proliferation and differentiation and leads to the upregulation of adhesion molecules in endothelial cells. TNF-α is also involved in apoptosis via recruitment and autoproteolytic activation of caspases (Ndebele *et al.,*2008).

A study by Mohamed and Altaii (2020), showed elevated levels of TNF-α in rate's serum after injecting them with lipopolysaccharide (LPS) extracted from *E. coli*. An increased level of TNF-α was observed in other studies with induced animal colitis ( Yang *et al*.,2014; Gupta *et al.,*2018). The results of this study came following a study by YU *et al*., (2018) in which they noticed that oral administration of microcin J25 (MccJ25) infected mice significantly increased the pro-inflammatory cytokines TNF-α secretion levels, as well as decreased IL1-0 concentrations compared to control group. TNF-α is important in the

pathogenic, both in mouse models of IBD and colitis-associated colon cancer, it induces an immunopathology in the colon of mice that is similar to that seen in CD-associated colitis (Jones-Hall & Nakatsu,2016). Anti-TNF-α therapy is systemically administered and effective in the treatment of IBD. (Gareb *et al*.,2020). Many anti-TNF-α therapies are used in the clinical setting of IBD such as infliximab, adalimumab, golimumab, certolizumab, etanercept, etanercept, and CDP571. These biologicals are antibodies or soluble TNF-α receptors that neutralize TNF-α (Gareb *et al*.,2020).

**3-8-1-3 IL-10/TNF-α Ratio**

To understand the mechanism of the work of pro- and anti-inflammatory cytokines *in vivo*, the ratio between IL-10 and TNF-α in the rat groups was calculated and illustrated in Figure 3-10, In the control group the level of IL-10 higher than TNF-α the ratio was 7.95, whereas the ratio in the groups that orally given *E. coli* with microcinB17 gene isolated from UC patients was 5.24 which means, TNF-α elevated more theIL-10. In the third group of rats, *E. coli/+ McbA* isolated from CD the ratio was 7.22. Whereas in the fourth and fifth groups of rats with *E. coli/- McbA* isolated from UC and CD patients 6.59,7.29 respectively, which means the two cytokines elevated together.

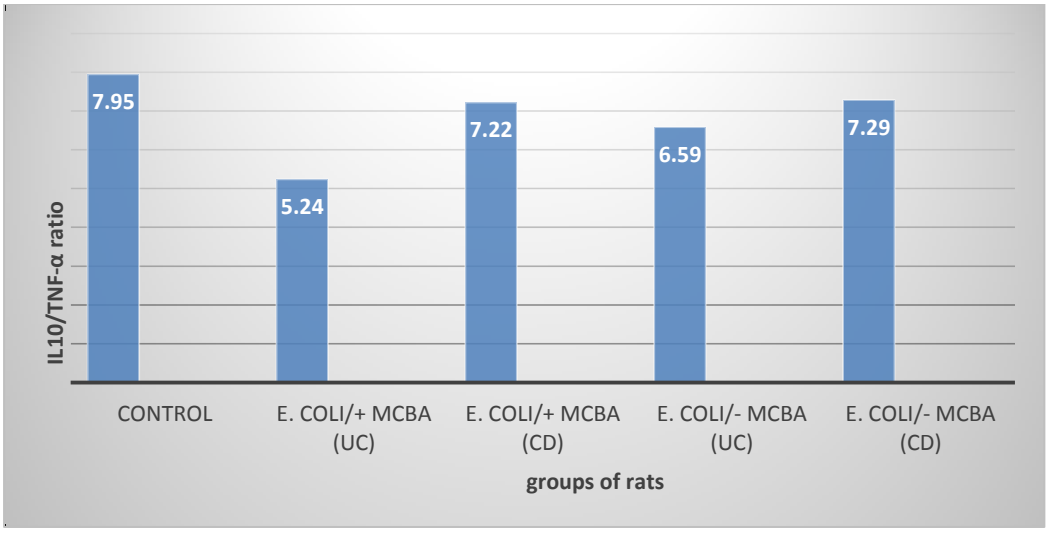



**Figure 3-10: IL-10/TNF-α ratio**

The investigation of the IL-10 / TNF-α ratio is important in predicting the hypersusceptibility to infections, serves as a surrogate measure of pro- to anti-inflammatory immune homeostasis, and as a biomarker in many infections (Tsurumi *et al*.,2016). The IL-10/TNF-α ratio has been used as a biomarker in a few cohort studies. A study among north Indian adults found it to be elevated in the plasma of acute myocardial infarction patients compared to that of matched healthy control (Goswami *et al*.,2009). An additional study conducted in Brazil the TNF-α/IL-10 ratio to be correlated with hyperglycemia in the plasma and placenta of pregnant women (Moreli *et al*.,2015). While in a study by Tsurumi *et al*.,(2016) demonstrated the use of the TNF-α/IL-10 ratio as a biomarker in the context of trauma and infection prediction.

## 3-8-2 Measurement of Blood Parameters in Rat's Groups:

There was a significant increase in the count of WBC (P ≤0.01) and lymphocytes (P≤0.05) among all studied groups Table 3-10 and 3-11 respectively. There were significant differences in the numbers of WBC between group I (3.12) $10^3$ /µL and group II (15.42) $10^3$ /µL whereas in the number of lymphocytes between group I (2.10) $10^3$ /µL and group II (6.96) $10^3$ /µL. Appendix 5,6

**Table 3-10: The Comparisons among under-studied rat groups based on WBC.**

| Groups | Mean $10^3$ /µL | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| V | 5.74 | 1.14 | 0.51 | 0.003** |
| IV | 7.04 | 0.81 | 0.36 | |
| III | 7.06 | 2.28 | 1.02 | |
| II | 15.42 | 4.99 | 2.81 | |
| I | 3.12 | 2.00 | 0.89 | |

Kruskal-Wallis tests have been utilized to discern statistically significant differences across multiple independent groups.
-*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.
**I: control, II: rats dosed *E. coli/+ McbA* isolates from UC, III: rats dosed *E. coli/+ McbA* isolates from CD, IV: rats dosed *E. coli/- McbA* isolates from UC, V: rats dosed *E. coli/- McbA* isolates from CD**

**Table 3-11: The Comparisons among under-studied rat groups based on Lymphocytes**.

| Groups | Mean $10^3$ /µL | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| V | 4.52 | 2.49 | 1.11 | 0.033* |
| IV | 5.38 | 2.45 | 1.10 | |
| III | 3.38 | 1.65 | 0.74 | |
| II | 6.96 | 2.87 | 1.28 | |
| I | 2.10 | 1.87 | 0.83 | |

ANOVA tests have been utilized to discern statistically significant differences across multiple independent groups.
-*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

**I: control, II: rats dosed *E. coli/+ McbA* isolates from UC, III: rats dosed *E. coli/+ McbA* isolates from CD, IV: rats dosed *E. coli/- McbA* isolates from UC, V: rats dosed *E. coli/- McbA* isolates from CD**

Hematological parameters such as red blood corpuscles (RBC) white blood cell (WBC) counts and hemoglobin (Hb) concentration are tightly regulated traits with high clinical relevance. Values outside normal ranges are diagnostic for disorders, including cancer, immune diseases, and inflammation (Kelada *et al*.,2012).

The analysis of components of the CBC is a simple and inexpensive method to assess the disease activity of IBD. The association between CBC parameters and disease activity in UC and CD patients has previously been studied (Cherfane *et al*.,2015; Acartetrk *et al*.,2015).

Leucocytes are significantly less numerous than erythrocytes, yet their numbers rise during an infection. Leucocytes, which were classified as granulocytes (neutrophils, eosinophils, and basophils) or agranulocytes (monocytes and lymphocytes), can recognize foreign material and either engulf cells or emit membrane-disrupting substances that can kill the organism. Lymphocytes are vital in the immune response to diseases because they monitor the internal environment and produce antibodies against infections (Glenn & Armstrong, 2019). It has been shown in a study by Chen *et al*.,(2020) that in patients with active UC and CD, there was an increase in the number of WBC and a decrease in the HGB compared to UC and CD patients in remission state. In another study where the percentage of lymphocytes in induced rat colitis was 63% compared to the control 45% (Patra *et al*.,2023).

Table 3-12 and Table 3-13 illustrated the comparisons between rat groups based on RBC and HGB. The mean count of RBC in the control group I was 7.58 $10^6$/µL, while in the other groups II, III, IV, and V were (3.86,4.82,5.18,4.14) $10^6$/µL respectively, which means there were significant differences between these groups (P ≤0.01). However, the comparisons between these groups in the mean of HGB were statistically significant differences at P≤0.05, Appendix 7,8

**Table 3-12: The comparisons between groups are based on RBC.**

| Groups | Mean $10^6$/µL | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| V | 4.12 | 1.60 | 0.72 | 0.005** |
| IV | 5.18 | 1.59 | 0.71 | |
| III | 4.82 | 1.91 | 0.86 | |
| II | 3.86 | 0.85 | 0.38 | |
| I | 7.58 | 1.03 | 0.46 | |

ANOVA tests have been utilized to discern statistically significant differences across multiple independent groups.
*. The mean difference is significant at the 0.05 level.

**. The mean difference is significant at the 0.01 level.

**I: control, II: rats dosed *E. coli/+ McbA* isolates from UC, III: rats dosed *E. coli/+ McbA* isolates from CD, IV: rats dosed *E. coli/- McbA* isolates from UC, V: rats dosed *E. coli/- McbA* isolates from CD**

**Table 3-13: The comparisons between groups in based on HGB.**

| Groups | Mean g/dL | Std. Deviation | Std. Error | P-value |
|---|---|---|---|---|
| V | 7.28 | 3.83 | 1.71 | 0.040* |
| IV | 6.72 | 2.47 | 1.11 | |
| III | 7.56 | 2.70 | 1.21 | |
| II | 7.20 | 2.72 | 1.22 | |
| I | 12.00 | 1.70 | 0.76 | |

ANOVA tests have been utilized to discern statistically significant differences across multiple independent groups.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.
**I: control, II: rats dosed *E. coli/+ McbA* isolates from UC, III: rats dosed *E. coli/+ McbA* isolates from CD, IV: rats dosed *E. coli/- McbA* isolates from UC, V:: rats dosed *E. coli/+ McbA* isolates from CD, IV: rats dosed *E. coli/- McbA* isolates from CD**

The decrease in the number of RBC and hemoglobin indicated the beginning of anemia which is correlated with the increased production of pro-inflammatory cytokines, such as IL-6, IL-17, and TNF-α, these cytokines cause anemia either by increasing hepcidin expression (a liver-derived peptide regulator of iron homeostasis) Hepcidin, in turn, acts as a negative regulator of intestinal iron absorption and macrophage iron release (Andrews *et al*.,2004) or by decrease expression of erythropoietin (Erythropoietin is a hormone that kidneys naturally make to stimulate the production of red blood cells). The treatment with anti-TNF-α agents has been shown to improve iron deficiency by improving erythropoiesis (Mahadea *et al*.,2021). These results came in consistence with two studies where the count of RBC and hemoglobin in induced colitis mice was less than control ( Herias *et al*., 2005; Patra *et al*.,2023).

## 3-8-3 Histological Changes in the Rats Intestine:

To confirm the correlation between the specific *E. coli* isolates and IBD, the experiment was designed to induce IBD in rats via oral administration of *E. coli* strains isolated from IBD patients. These attempts were consistent with Koch's postulates that are widely used to establish the causal relationship between a microbe and a disease. Other studies indicated that animal models are important in IBD experiments. The gastrointestinal tract is a highly complex organ in which multiple dynamic physiological processes are tightly coordinated while interacting with a dense and extremely diverse microbial population**,** there are many factors influencing the experimental host-microbe interaction in the gut, such as the density and diversity of microbiota and the innate and adaptive immunity (Parker *et al.*,2018). Consequently, germ-free and genetically altered animals, are the most animals used in gastrointestinal tract experiments (Gancarcikova *et al*.,2020; Harrison *et al*.,2018).

In the current study, white female rats were used. The assessment of infection in the intestine was done macroscopically and microscopically, macroscopically parameters such as hyperemia, adhesions, ulceration, and megacolon Microscopic features like abnormalities of mucosal architecture, crypt abnormalities, and inflammatory cell infiltration were assessed to describe the inflammatory status.

Figure 3-11 and Figure 3-12 showed the normal structure appearance of the small and large intestines of the control rats after administration of 1.5ml of normal saline.


**Figure 3-11: Histological sections of the small intestine of uninfected rats (control) showing a normal structure appearance of villi (V) and normal submucosa(SM), H & E stain by using magnification 10X**

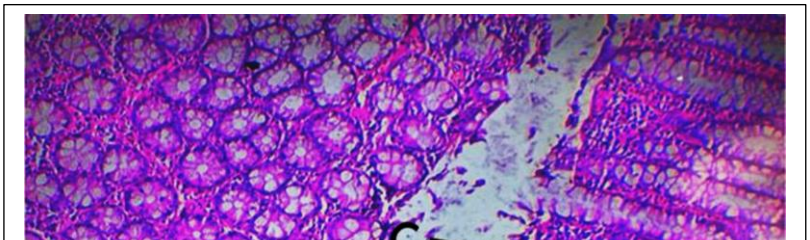


**Figure 3-12: Histological sections of the large intestine of uninfected rats (control) showing a normal structure of crypts (C), H & E stain by using magnification 10X**

Microscopically, the normal small intestine mucosa is distinguished by folds (plicae circulares, or kicking valves) and villi. Mucosa and submucosa make up the mucosal folds. Villi are mucosal folds that decrease in size from the proximal to the distal small intestine. Normal villus height is 0.5 to 1.5 mm; villus height should be greater than half the overall thickness of the mucosa and 3 to 5 times the length of the crypts. Enterocytes, goblet cells, and enteroendocrine cells line the villi. The most noticeable feature of the large intestine mucosa lack of villi. Instead, there are an extensive number of crypts that stretch deep and open onto a flat luminal surface. The stem cells that support rapid and continuous epithelium regeneration are found at the bottom or halfway down the crypts. These cells divide and proliferate to form the cryptal and surface epithelium. Goblet cells that secrete mucus are likewise significantly more common in the colonic epithelium than in the small gut (Bass & Wershil, 2016).

In the rat group that was orally given (*E. coli*/+ *McbA*) isolated from UC patient's stool, a section of the small intestine was illustrated in Figure 3-13. There were no noticeable changes in it with normal villi and submucosa.

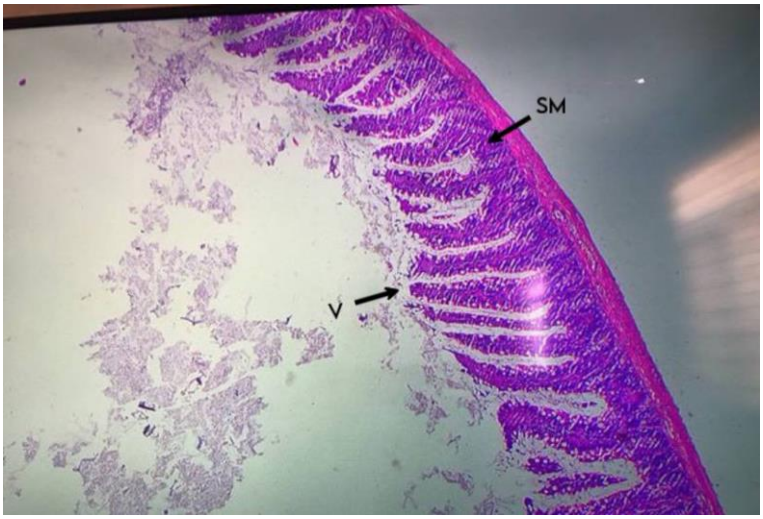
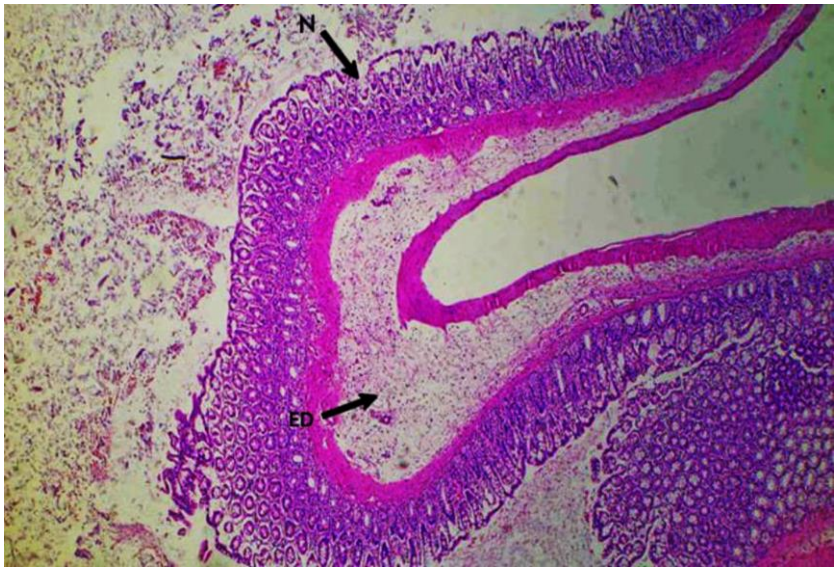


**Figure 3-13: Histological sections of the small intestine of infected rats with (*E. coli*/+ *McbA*) isolated from UC patient's stool showing a normal structure appearance of villi (V) and normal submucosa (SM), H & E stain by using magnification 4X.**

The bacterial populations in small intestine have lower biomass and are less diverse compared to large intestine because the harsh environment for microbial life owing to short transit time, the influx of digestive enzymes and bile, and intermittent food substrate delivery (Kastl Jr *et al*.,2020) maybe the reason for the poor attachment of pathogenic *E. coli* in the epithelial of small intestine since the attachment is the important facts or in the beginning of pathogenesis (Jackson & Fu, 2013).

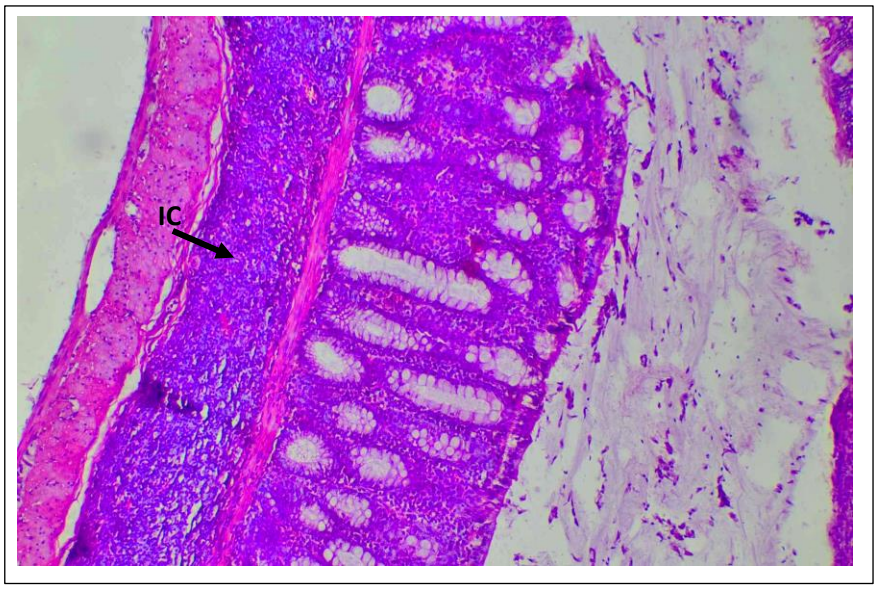
In the large intestine, there was the development of congestion with inflammatory cell infiltration in mucosa and submucosa, Figure 3-14 (A)(B)(C).



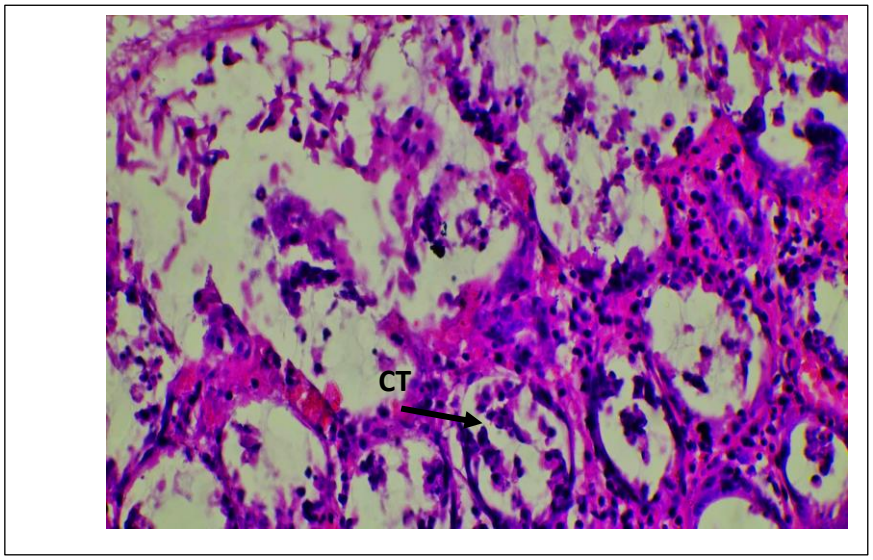

**A**

**Figure 3-14: Histological sections of the large intestine of infected rats with (*E. coli*/+ *McbA*) isolated from UC patient's stool showing necrosis (N) with edema(ED) in figure (A) inflammatory cell (IC) infiltration in the submucosa in**

**figure (B) and cryptitis (CT) in figure (C), H & E stain by using magnification 4X(A), 10X (B) and 40X (C)**




**B**




**C**

Studies on the of pathogenic *E.coli* in UC (obtained from UC patients' stool specimens and rectal biopsies) indicated that *E. coli* isolates adhered more strongly to buccal epithelial cells, causing mucosal damage similar to that seen with enteropathogenic *E. coli* (EPEC) (Hussein *et al*., 2008).

A study by Hussein *et al*., (2008) indicated that rats infected with 200 µL suspension containing $4 \times 10^8$ CFU of EPEC caused severe, diffuse, exceeding the submucosa and severe atrophy, branched crypts, cryptitis, crypt abscess with infiltration of cells in the large intestine.

Anatomically, the inflammation in UC was only present in the mucosa and submucosa of the large intestine with cryptitis and crypt abscesses, in contrast to the thickened submucosa, transmural inflammation, fissuring ulceration, and granulomas that were histologically present in CD. (Hoter& Naim, 2019).

Figure 3-15 illustrated the pathological changes in the small intestine from experimentally infected rats with (*E. coli*/+ *McbA*) isolated from CD patients representing normal small intestine. While the pathological changes in the large intestine included mild to moderate inflammation accompanied by reactive lymphoid tissue, Figure 3-16 (A)(B).

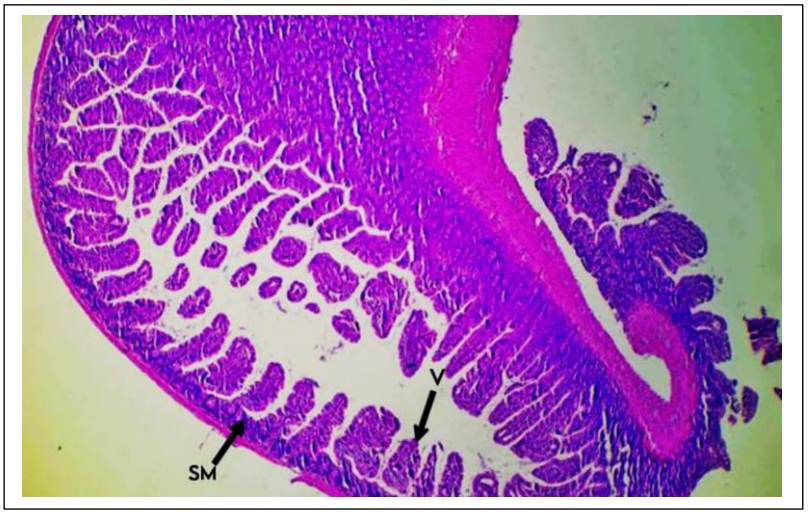
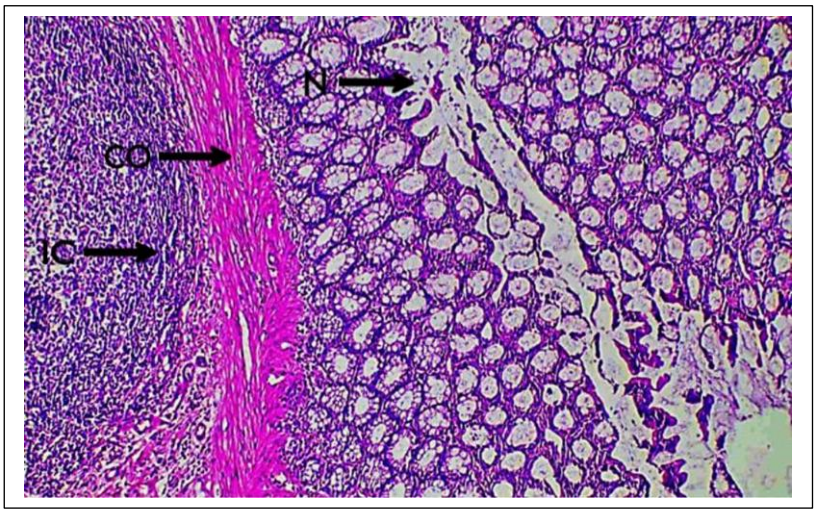
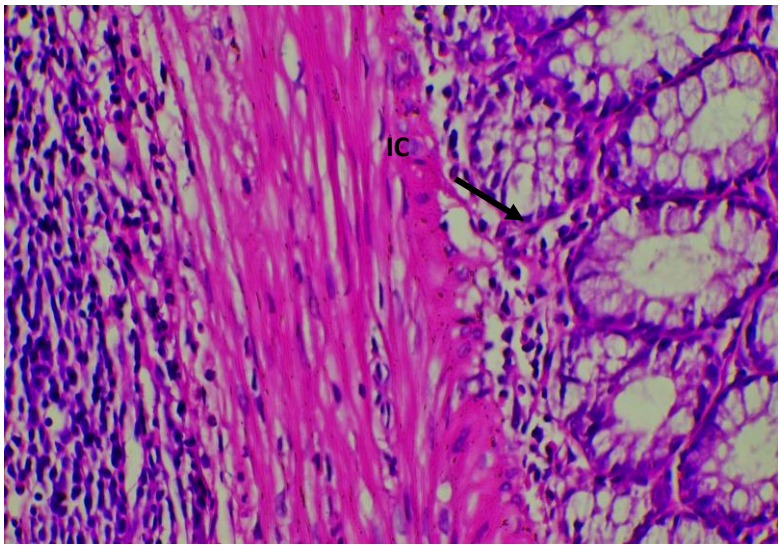



**Figure3-15: Histological sections of the small intestine of infected rats with (*E. coli*/ *McbA* $^{+}$) isolated from CD patient's stool showing a normal structure appearance of villi (V) and normal mucosa (M), H & E stain by using magnification 10X**




**A**

**Figure 3-16: Histological sections of the large intestine of infected rats with (*E. coli*/ $^{+}$*McbA*) isolated from CD patient's stool showing necrosis(N), congestion(CO), and inflammatory cell(IC), H & E stain by using magnification 10X (A) and 40X (B)**




**B**

The effect of administered *E. coli* which was isolated from CD patients appeared clear in the rat's large intestine although this disease usually involves and affects any region of the intestine in a discontinuous pattern (Guan,2019).

In many studies, CD was associated with virulent adherent invasive *E. coli* bacteria, via expression of flagella, which can potentiate an inflammatory mucosal immune response involving increased expression of toll-like receptor (TLR5) (Carvalho *et al*.,2008) or induce inflammation by adhering to an N-glycosylated chitinase via the chitin-binding domain (Low *et al*.,2013).

MicrocinB17 as an oxazole structure was sufficient to induce intestinal inflammation *in vivo*. Intra-rectal administration of mice leads to increased weight loss and pathology characterized by superficial inflammation of the gut wall, neutrophil accumulation, and ulceration of the epithelial layer of the colon (Iyer *et al*.,2018).

There are many studies where the effect of microcin was distinct in experimental models such as a study by Yu *et*.,(2018) revealed that microcin showed effects and changes in the intestine of the mice treated with 18.2 mg/kg of microcin J25 (MccJ25) which was significantly reduced the V/C length in jejunum compared with control group a marked influence on the villous height (V) and crypt depth (C) and the V/C in the jejunum, this toxin effect also on the distribution of the normal flora of the large intestine by increased coliform bacteria and *Clostridium spp*. numbers and lower Bifidobacterium numbers than the control group (Yu *et al*.,2018). Another study by Hammad & Obaid, (2020) revealed the genotoxic effects of crude bacteriocin were detected on albino mice bone marrow cells *in vivo*, The results showed an acute dose-dependent toxic effect of the crude bacteriocin; The higher doses (150 and 300 mg/kg) caused a significant increase in the micronuclei frequency in the

bone marrow cells. Furthermore, DNA damage increased significantly and proportionally to higher bacteriocin doses**.** Purified toxin was used in these two studies and that may be the cause of its effectiveness.

Figures 3-17 and 3-18 illustrated the histological sections prepared from the small intestine of the rat's administration with *E. coli*/- *McbA* isolated from UC and CD patients respectively, were showed normal with the well-preserved structure of villi and no significant changes could be noticed. These results might indicated that either *E. coli*/- *McbA* strains cannot induce inflammatory changes in the small intestine or those strains require a longer period to exert their effect on it.

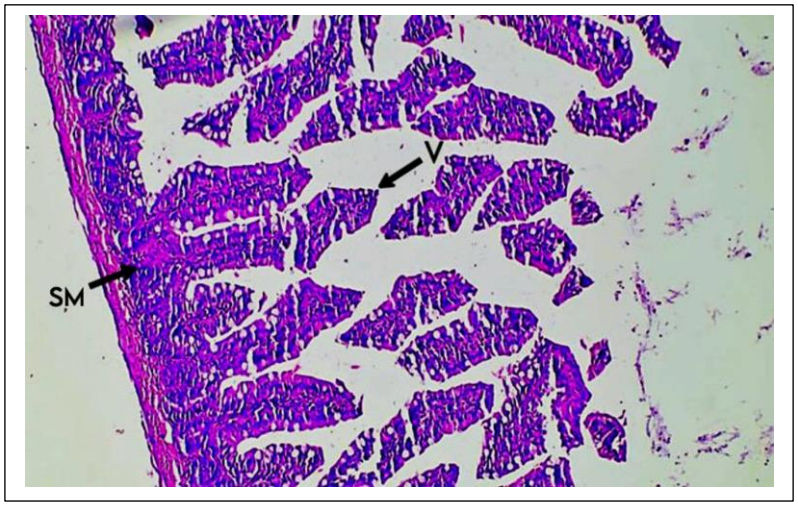



**Figure 3-17: Histological sections of the small intestine of orally administered rats with (*E. coli*/- *McbA*) isolated from UC patients stool showing normal villi(V) and submucosa (SM), H & E stain by using magnification 10X**

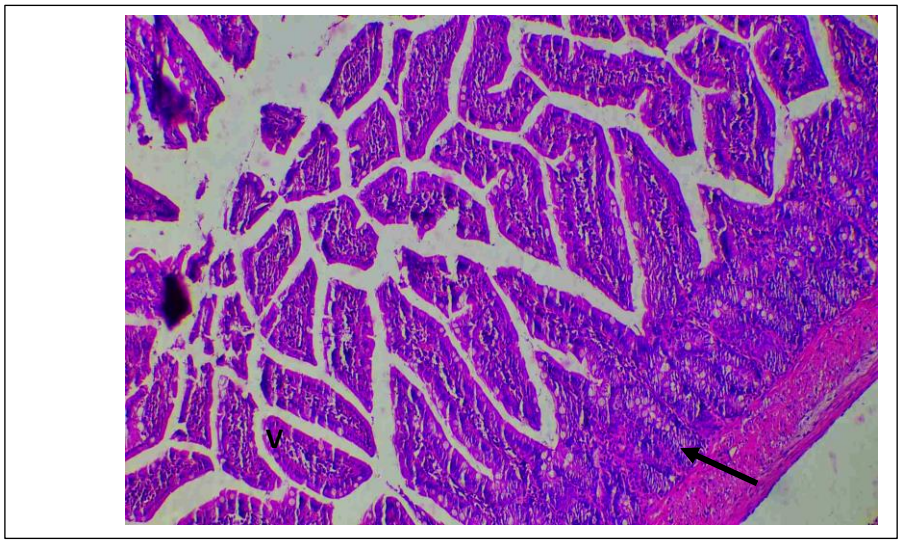


**Figure 3-18: Histological sections of the small intestine of orally administered rats with (*E. coli*/- *McbA*) isolated from CD stool showing normal villi(V) and submucosa (SM), H & E stain by using magnification 10X**

In contrast to the small intestine, histological examination of sections of the large intestine of those rats revealed significant microscopic changes represented by congestion in the submucosa, granulocyte infiltration with inflammation, and the beginning of necrosis in the mucosa. In addition, the crypts appeared irregular and dilated, Figure 3-19,3-20(A)(B)(C).

**Figure 3-19: Histological sections of the large intestine of orally administered rats with (*E. coli*/ -*McbA*) isolated from UC stool showing necrosis(N) in the mucosa and congestion(CO) in sub mucosa, H & E stain by using magnification 10X**

**Figure 3-20: Histological sections of the large intestine of orally administered rats with (*E. coli/ -McbA*) isolated from CD patient's stool showing edema (ED) in the submucosa(A), necrosis (N) (B) inflammatory cell (IC) in(C)ss, H & E stain by using magnification 4X(A), 10X(B), 40X(C).**

Macroscopically, the descending colon in all infected rats appeared swollen with edema. In a study by Mirsepasi-Lauridsen *et al*.,(2020) they used two types of mice; genetically altered and wild type to colonize them with alpha-hemolysin-producing *E. coli*, they found that infected genetically altered and wild-type mice showed increased inflammation/increased leucocytes, the thickness of smooth muscles and initiation to ulcerative formation in the ileum and cecum of the infected mice, to a higher degree in genetically altered than in wild type mice. *E. coli* pathotypes elicit their pathogenicity in the large intestine through adhesion, invasion, and/or toxin production, these strains are mucosa-associated with many virulence factors such as type 1 fimbriae**,** cytotoxic necrotizing factor, aerobactin synthesis and colicin B determinants (Micenková *et al*.,2018; Abdelhalim *et al*.,2020).

# *Recommendation & Conclusions*

## Conclusions

### The present study has reached the following conclusions:

1. Crohns disease was the subtype of IBD that is prevalent among young patients, whereas ulcerative colitis was the subtype that was most prevalent among elderly patients.
2. *E. coli* isolates from IBD patient's stool showed a high prevalence of The toxin microcin B17.
3. All *E. coli* isolates from IBD patient's stools (whether have microcin B17gene or not) were able to induce inflammatory responses and histological changes in the intestines of rats.

### Recommendations

1. Further studies are needed to identify *E. coli* and other virulence factors that may be responsible for IBD and to determine the physiopathology of these infections to consider possible prevention measures and means.
2. Extraction of microcinB17 toxin from isolated bacteria is recommended for further studies in vivo and in vitro to demonstrate its role in IBD pathology.

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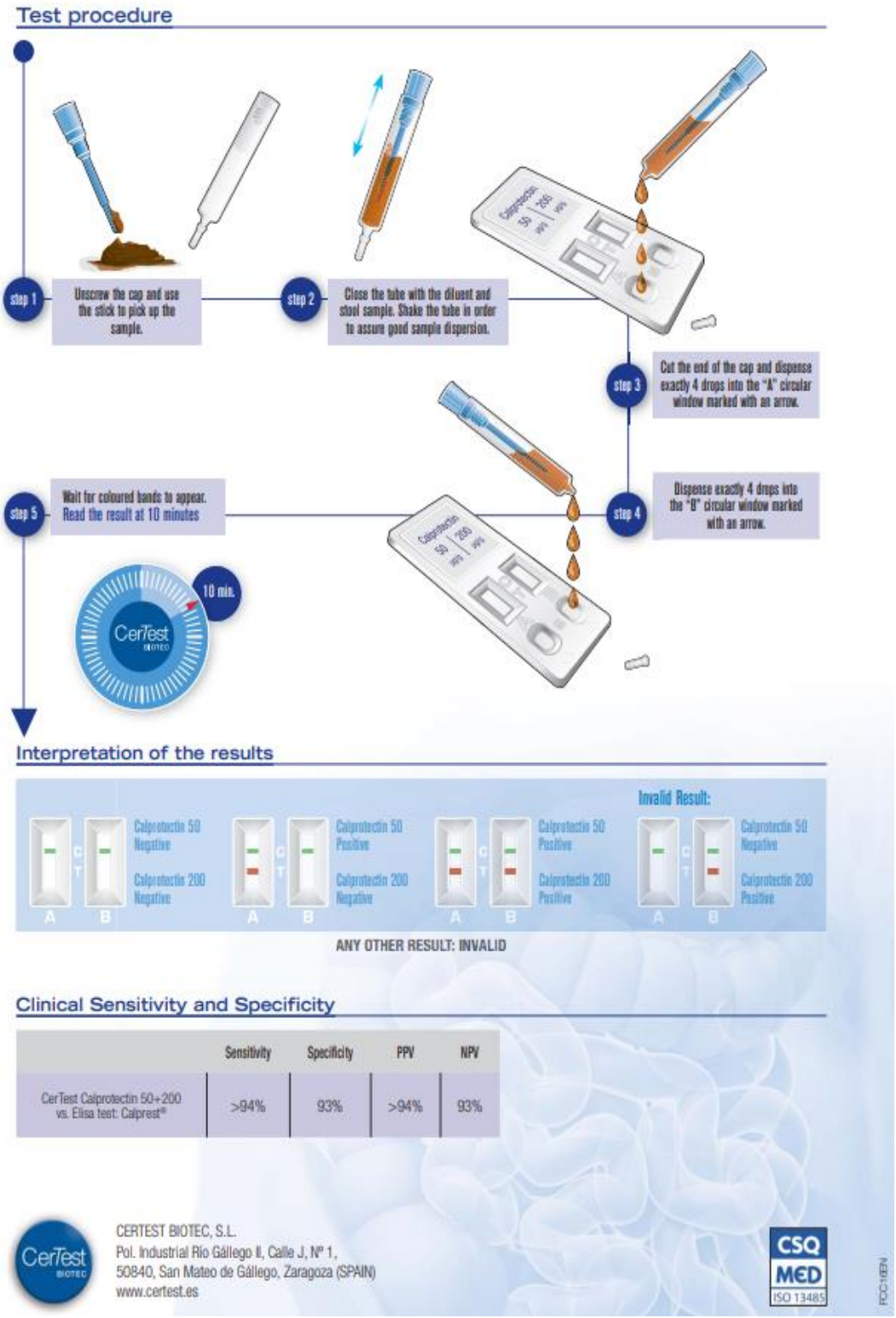
# *Appendix*

Appendix 1: The questionnaire paper used in the study

| **The questioner paper** | |
|---|---|
| 1. **Type of IBD disease** | **6. type of treatments** |
| ○ Crohn disease | **7. type of food** |
| ○ Ulcerative colitis | ○ meat |
| **2. Gender** | ○ sweet |
| ○ male | ○ vegetable |
| ○ female | |
| **3. Age** | **8. smoking** |
| ○ 1-10 | ○ yes |
| ○ 11-20 | ○ no |
| ○ 21-30 | **9. sport** |
| ○ 31-40 | ○ yes |
| ○ 41-50 | ○ no |
| ○ 51-60 | **10. If there is another member in the family with the same symptoms** |
| ○ 61-70 | ○ yes |
| **4. Symptoms of disease** | ○ no |
| ○ diarrhea | **11. The weight** |
| ○ constipation | **12. The place you live** |
| ○ blood with stool | |
| ○ nausea | |
| **5. Extraintestinal symptoms** | |
| ○ arthritis | |
| ○ eye infection | |
| ○ liver infection | |
| ○ skin infection | |
| ○ fistula | |

Appendix 2: the procedure of the calprotectin test



Appendix 3:Hematology data of female rats, aged 8-18 weeks

| TEST | UNIT | N | MEAN | S.D. | RANGE |
|---|---|---|---|---|---|
| MPV | fL($\mu m^3$) | 176 | 7.8 | 0.9 | 6.2-9.8 |
| Platelets | $10^3/\mu L$ | 175 | 929 | 133 | 680-1200 |
| RDW | % | 176 | 12.2 | 1.2 | 10.5-14.9 |
| MCHC | g/dL | 176 | 35.3 | 1.3 | 33.2-37.9 |
| MCH | pg | 176 | 19 | 0.8 | 17.8-20.9 |
| Hematocrit | % | 176 | 43.3 | 3.1 | 37.9-49.9 |
| Hemoglobin | g/dL | 176 | 15.2 | 0.9 | 13.7-16.8 |
| Red Blood Cells | $10^6/\mu L$ | 176 | 8.02 | 0.53 | 7.07-9.03 |
| White Blood Cells | $10^3/\mu L$ | 176 | 3.12 | 1.49 | 1.13-7.49 |
| MCV | fL($\mu m^3$) | 176 | 53.8 | 2.3 | 49.9-58.3 |
| Neutrophils | $10^3/\mu L$ | 174 | 0.46 | 0.31 | 0.15-1.5 |
| Lymphocytes | $10^3/\mu L$ | 176 | 2.5 | 1.21 | 0.82-5.66 |
| Monocytes | $10^3/\mu L$ | 175 | 0.06 | 0.03 | 0.02-0.16 |
| Eosinophils | $10^3/\mu L$ | 175 | 0.05 | 0.03 | 0.01-0.15 |
| Basophils | $10^3/\mu L$ | 176 | 0.01 | 0.01 | 0-0.03 |
| Reticulocytes | $10^9/L$ | 156 | 216.6 | 57 | 129.8-383.7 |
| CHCM | g/dL | 176 | 35.7 | 1.3 | 33.3-38.1 |
| CH | pg | 176 | 19.2 | 0.7 | 18.1-20.9 |
| HDW | g/dL | 176 | 2.31 | 0.25 | 1.88-2.81 |
| PDW | % | 176 | 52.7 | 5.5 | 42.2-64.4 |
| % Neutrophils | % | 175 | 15.4 | 6.4 | 7.1-33.2 |
| % Lymphocytes | % | 175 | 80 | 6.7 | 62.2-90 |
| % Monocytes | % | 176 | 2 | 0.8 | 0.8-3.9 |
| % Eosinophils | % | 174 | 1.7 | 1 | 0.5-4.5 |
| % Basophils | % | 176 | 0.3 | 0.2 | 0-0.8 |
| % Large unstained cells | % | 171 | 0.5 | 0.2 | 0.1-0.9 |
| Large unstained cells | $10^3/\mu L$ | 172 | 0.01 | 0.01 | 0-0.04 |
| % Reticulocytes | % | 156 | 2.7 | 0.8 | 1.7-4.7 |

Appendix 4 :Multiple Comparison of TNF-α

Table 1.1: Multiple Comparisons Within Groups Based on TNF.

| Groups | P-value |
|---|---|
| III -V | 0.995 |
| III - IV | 0.912 |
| III - II | 0.003** |
| III - I | 0.598 |
| V - III | 0.995 |
| V- IV | 0.729 |
| V- II | 0.001** |
| V- I | 0.819 |
| IV- III | 0.912 |
| IV- V | 0.729 |
| IV- II | 0.023* |
| IV- I | 0.183 |
| II- III | 0.003** |
| II -V | 0.001** |
| II- IV | 0.023* |
| II - I | 0.001** |
| I - III | 0.598 |
| I - V | 0.819 |
| I - IV | 0.183 |
| I - II | 0.001** |

Tukey's Honest Significant Difference (HSD) post hoc test has been utilized for conducting multiple comparisons.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

Appendix 5:Multiple comparisons of WBC

Table 2.1: Multiple Comparisons Within Groups based on WBC.

| Groups | P-value |
|---|---|
| III - V | 0.997 |
| III - IV | 1.000 |
| III - II | 0.219 |
| III- I | 0.835 |
| V- V | 0.997 |
| V- IV | 0.997 |
| V- II | 0.119 |
| V- I | 0.956 |
| IV- III | 1.000 |
| IV- V | 0.997 |
| IV- II | 0.217 |
| IV- I | 0.837 |
| II - III | 0.219 |
| II - V | 0.119 |
| II - IV | 0.217 |
| II - I | 0.001* |
| I- III | 0.835 |
| I - V | 0.956 |
| I- IV | 0.837 |
| I - II | 0.001* |

Tukey's Honest Significant Difference (HSD) post hoc test has been utilized for conducting multiple comparisons.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

Appendix 6:Multiple comparisons of lymphocyte

Table 3.1: Multiple Comparisons Within Groups based on Lymphocyte.

| Groups | P-value |
|---|---|
| III - V | 0.933 |
| III - IV | 0.653 |
| III - II | 0.142 |
| III - I | 0.902 |
| V- V | 0.933 |
| V- IV | 0.975 |
| V- II | 0.473 |
| V- I | 0.481 |
| IV- III | 0.653 |
| IV- V | 0.975 |
| IV- II | 0.814 |
| IV- I | 0.204 |
| II - III | 0.142 |
| II - V | 0.473 |
| II - IV | 0.814 |
| II - I | 0.025* |
| I - III | 0.902 |
| I - V | 0.481 |
| I - IV | 0.204 |
| I - II | 0.025* |

Tukey's Honest Significant Difference (HSD) post hoc test has been utilized for conducting multiple comparisons.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

Appendix 7:Multiple comparison of RBC

Table 4.1: Multiple Comparisons Within Groups based on RBC.

| Groups | P-value |
|---|---|
| III - V | 0.938 |
| III - IV | 0.995 |
| III - II | 0.831 |
| III - I | 0.049* |
| V- V | 0.938 |
| V- IV | 0.776 |
| V- II | 0.998 |
| V- I | 0.009 |
| IV- III | 0.995 |
| IV- V | 0.776 |
| IV- II | 0.612 |
| IV- I | 0.105 |
| II - III | 0.831 |
| II - V | 0.998 |
| II - IV | 0.612 |
| II - I | 0.005** |
| I - III | 0.049* |
| I - V | 0.009** |
| I - IV | 0.105 |
| I - II | 0.005** |

Tukey's Honest Significant Difference (HSD) post hoc test has been utilized for conducting multiple comparisons.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

Appendix 8: Multiple Comparisons of HGB

Table 5.1: Multiple Comparisons Within Groups based on HGB.

| Groups | P-value |
|---|---|
| III - V | 1.000 |
| III - IV | 0.988 |
| III - II | 1.000 |
| III - I | 0.122 |
| V- V | 1.000 |
| V- IV | 0.998 |
| V- II | 1.000 |
| V- I | 0.091 |
| IV- III | 0.988 |
| IV- V | 0.998 |
| IV- II | 0.999 |
| IV- I | 0.048* |
| II - III | 1.000 |
| II - V | 1.000 |
| II - IV | 0.999 |
| II - I | 0.083 |
| I - III | 0.122 |
| I - V | 0.091 |
| I - IV | 0.048* |
| I - II | 0.083 |

Tukey's Honest Significant Difference (HSD) post hoc test has been utilized for conducting multiple comparisons.
*. The mean difference is significant at the 0.05 level.
**. The mean difference is significant at the 0.01 level.

**الملخص**

تعد أمراض التهاب الامعاء (Inflammatory bowel disease, IBD) من الامراض المزمنة التي تصيب القناة الهضمية والتي تشمل كل من مرض كرونز( Crohn's disease, CD) ومرض القولون التقرحي (Ulcerative colitis, UC).

تم في هذه الدراسة جمع 60 عينة خروج من اشخاص مصابين بأمراض التهاب الامعاء (IBD ) والتي شملت 30 عينه من مرضى كرونز(CD ) و30 عينه من مرضى القولون التقرحي (UC ) من مختلف الفئات العمرية ومن كلا الجنسين. وقد جمعت العينات من المرضى المراجعين لمركز الجهاز الهضمي وامراض الكبد في محافظة كربلاء في الفترة ما بين شهر اب 2021 الى شهر ايلول 2022 . بألاضافة الى أنه تم جمع 30 عينة خروج من اشخاص أصحاء غير مصابين للمقارنة. كل المرضى كانوا في حالة التهاب نشط والذي تم اثباته من خلال اجراء أختبار الكالبروتكتين لهم (Fecal calprotectin, FC) .

وقد لوحظ ان نسبة الأناث المصابات بهذا المرض 60% وهي أعلى من الذكور 40% فيما لوحظ أن أعمار المرضى الذين يعانون من مرض التهاب الأمعاء كان مختلفًا مع وجود فروقات أحصائية معنوية بينهم ، أشارت النتائج إلى أن مرض كرونز (CD) هو النوع الفرعي من مرض التهاب الأمعاء (IBD )السائد بين المرضى ضمن الاعمار الصغيرة والشباب، في حين أن مرض القولون التقرحي (UC) هو النوع الأكثر انتشارًا بين المرضى ألأكبرعمرا . وعند ملاحظة اهم الاعراض عند المرضى نجد ان الاسهال هو الاكثر شيوعا في كلا النوعين وخاصة الاسهال المصحوب بالدم عند مرضى القولون التقرحي (UC ).وقد صاحب المرضى ايضا إضطرابات خارج معوية مثل التهاب المفاصل وتقرحات جلدية واضطراب في العين.

ان بكتريا الاشيريكية القولونية (*Escherichia Coli*) الممرضة كانت من أكثر ألانواع البكتيرية المعزولة من عينات خروج الاشخاص المصابين بأمراض التهاب الامعاء (IBD) وان هذه البكتريا تمتازبأمتلاكها للعديد من عوامل الضراوة ومن ضمنها انتاج السموم

مثل سموم الكوليبكتين (colibactin ) وسموم المايكروسين بي 17 (microcin B17 ) والتي اثبتت الدراسات الحديثة علاقتها مع تطورهذه الامراض.

تم عزل و تشخيص بكتريا الاشيريكية القولونية من عينات خروج المرضى بأستخدام طرق العزل والتشخيص التقليدية ثم تم تأكيد التشخيص باستعمال تقنية تفاعل البلمرة المتسلسل (PCR ) من خلال الكشف عن الجين التشخيصي للبكتريا ( *16s rRNA* ) وباستعمال نفس التقنية تم الكشف عن جينات انتاج السموم في البكتريا المعزولة شملت كل من جين سموم الكوليبكتين (*clbB* ) وجين سموم المايكروسين بي 17 (*McbA* ) وقد جاءت نتائج تضخيم جين سموم الكوليبكتين من 60 عزلة بكتيرية 10 عزلات فقط كانت تملك هذا الجين ( 4 من مرضى كرونز و6 من مرضى القولون التقرحي) في حين كانت أعداد العزلات التي تمتلك جين سموم المايكروسين بي 17 عالية في 52 من 60عزلة ( 27 عزلة من مرضى كرونز و 25 عزلة من مرضى القولون التقرحي).

من أجل اثبات دور سموم المايكروسين بي 17 في تطورامراض التهاب القولون تمت دراسة تأثيره على حيوانات مختبرية شملت 25 جرذ من الاناث البيض حيث تم تقسيمها الى خمس مجاميع كألاتي : المجموعة الاولى تم تجريعها فمويا من السائل المغذي للمقارنة والمجموعة الثانية تم تجريعها عالق بكتيري من البكتريا المعزولة من مرضى القولون التقرحي التي تملك جين انتاج السموم مايكروسين بي 17 (*E. coli*/+ *McbA* ) والمجوعة الثالثة تم تجريعها عالق بكتيري من البكتريا المعزولة من مرضى كرونز الحاوية على جينات سموم المايكروسين بي 17 (*E. coli*/+ *McbA* ) والمجموعة الرابعة تم تجريعها عالق بكتيري من البكتريا المعزولة من مرضى القولون التقرحي لا تمتلك جين انتاج السموم (*E. McbB* *coli*/- *McbA*) والمجموعة الخامسة تم تجريعها عالق بكتيري من البكتريا المعزولة من مرضى كرونزلا تمتلك جين انتاج السموم *McbB* (*E. coli*/- *McbA*). وبعد 15 أسبوع من التجريع بواقع 35 جرعة تم سحب الدم من المجاميع الاربعة من اجل تحديد مستوى الحركيات الخلوية interlukin-10 (IL-10) و tumor necrosis factor-alpha (TNF-α) و تم تشريح الجرذان من اجل عزل اجزاء من امعائهم الدقيقة والغليظة و عمل مقاطع نسيجية منها وتقييم التغيرات النسيجية فيها.

جاءت النتائج ان جميع المجاميع الاربعة من الجرذان التي تم تجريعها العالق البكتيري من البكتريا المعزولة من خروج مرضى التهاب الامعاء بنوعيه سواء كانت تملك جين السموم او لا قد أظهرت ارتفاعا ملحوظا في مستوى الحركيات الخلوية في دمها ولوحظ ان الامعاء الغليظة للجرذان قد تأثرت بالبكتريا وظهرت عليها بعض التغيرات النسيجية بينما لم تتأثر امعائها الدقيقة.

ومن ذلك نستنتج أن جميع عزلات الإشريكية القولونية التي تم عزلها من براز مرضى التهاب الأمعاء (سواء كانت تنتج ميكروسين B17 أم لا) كانت قادرة على إحداث استجابات التهابية وتغيرات نسجية في أمعاء الجرذان. وبناءً على ذلك، قد تلعب عوامل ضراوة أخرى (بالإضافة إلى الميكروسين B17 ) دورًا في التسبب في مرض التهاب الأمعاء(IBD) .

جامعة كربلاء

كلية العلوم

قسم علوم الحياة

# دراسة تأثير بكتريا *Escherichia coli* المنتجة لسموم Microcin B17 المعزولة من مرضى التهاب الأمعاء في اناث الجرذان

أطروحة

مقدمة الى كلية العلوم-جامعة كربلاء وهي جزء من متطلبات نيل درجة الدكتوراه

فلسفة في علوم الحياة

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بكلوريوس علوم حياة/كلية العلوم/جامعة بغداد(2001 )

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