Role of Intestinal Microbiota and its Virulence Factors in Pathogenesis of Inflammatory Bowel Disease

Sarah S. Abdul-Hussein[1], Neihaya H. Zaki[2], Ekhlass N. Ali[2], Ali H. Ad'hiah[3]

[1]Student, [2]Prof., Department of Biology, College of Science, Al-Mustansiryia University, Baghdad, Iraq, [3]Prof., Tropical-Biological Research Unit, College of Science, University of Baghdad

Abstract

Inflammatory bowel disease (IBD) is a chronic relapsing unexplained etiological condition and microbiota have been suggested to influence its etiology and pathogenesis. Ulcerative colitis (UC) and Crohn's disease (CD) are the two main categories of this disease. The present study examined the fifty fecal samples obtained from IBD patients and control. The bacterial isolates were identified by culture, microscope examination, biochemical test, and Vitek 2 compact system.Results showed that IBD patients havedifferent percentages of Proteobacteria (54.5%), Actinobacteria (1.6%), and Firmicutes (43.8%) when identified. They encompassed 17 genera that involve 121 bacterial isolates. The corresponding percentages in control were 49%, 0% and 51%, respectively, with four genera and 68 bacterial isolates. The bacterial isolates of IBD patients and control are assessed for some virulence factors, which included biofilm formation and production of phospholipase and hemolysin, as well as antibiotic susceptibility. In IBD patients, evaluation of biofilm formation revealed that 20.6% of isolates were high-producing, 69.4% was moderate producing and10% non-biofilm producing. The corresponding percentages in control were 1%, 62% and 37%, respectively. The bacterial isolates showed different abilities in producing phospholipase. In samples of patients, 20.5% of isolates showed large activity, 44% moderate activity and 35.5% negative activity. For control samples, most of the isolates were non-producer of phospholipase (66%), while large and moderate enzyme activity accounted for 4.5% and 29.5%, respectively. The hemolysin enzyme activity was determined as 35% of isolates with high activity, 40% with moderate activity and 25% without activity. These percentages in control isolates were 25%, 35% and 40%, respectively. Imipenem was the most effective antibiotic against gram-positive and gram-negative bacteria of the patient isolates with the resistance of 27% and 24% respectively, while the less effective antibiotics was cefixime with resistance of 95% and 88%, respectively. In the control isolates, the imipenem was also the most effective against bacteria with a resistance of 15%. The less effective antibiotics for gram-positive isolates was erythromycin with resistance of 43%and for gram-negative isolates, it was tetracycline was (45%).

Keywords: *Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Microbiota; Virulence factors, Dysbiosis.*

Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing unexplained etiological condition[(1)]. Ulcerative colitis (UC) and Crohn's disease (CD) are the two main categories of inflammatory bowel disease, these groups of chronic idiopathic inflammatory diseases affecting the digestive system[(2)]. The etiology of both subtypes is incompletely understood but it is suggested to involve complex interactions between genetic, environmental, immunological, and gut microbiomial factors. Their interactions orchestrate a cascade of inflammatory responses in the intestinal mucosa[(3)].

In humans more than 100 trillion microorganisms colonize the gastrointestinal tract creating mutual relations with the host which are collectively referred to as

Corresponding Author:
Sarah S. Abdul-Hussein
Student, Department of Biology, College of Science, Al-Mustansiryia University, Baghdad, Iraq
e-mail: sprincess455@gmail.com

the gut microbiota[4,5]. Such indigenous microorganisms co-evolved in a symbiotic relationship with the host. Beyond their metabolic advantages, symbiotic bacteria provided the host with several activities that encourage immune homeostasis, immune responses, and pathogen colonization inhibition. The capacity of symbiotic bacteria to prevent pathogen colonization is mediated through many mechanisms involving direct killing, competition for limited nutrients, and enhanced immune responses [5].

Metagenomic data suggest that the most predominant phyla in healthy individuals are in gram-negative Bacteroidetes (17–60%) and gram-positive firmicutes (35–80%)[6,7]. The intestinal microenvironment comprising gut microbiota and its metabolites, which is easily altered by nutrition, medications, stress, bacterial or viral pathogens infection[8,9].

Host immunity should adapt to change according to the gut environment, along with dysbiosis and infection with pathogenic bacteria. The imbalance between protective and harmful bacteria known as dysbiosis, resulting in loss of intestinal homeostasis, is described in numerous intestinal diseases including irritable intestinal syndrome (IBS) and inflammatory intestinal disease (IBD) [10,11]. Zuo and Ng, (2018) have been documented that the inflammatory environment in IBD supports the growth of adherent invasive bacterial strains such as Fusobacteria and Enterobacteriaceae. As well, an increased number of adherents invasive E. coli has been found in both UC and CD[13,14]. Recently, the microbiota uses as a biomarker to screen the progression of IBD and the specific strains require stimulating or treating IBD need further investigation[15].

Material and Method

Patients and Control: During January–June 2019, a case-control study was conducted on 50 IBD patients and 50 healthy control subjects after receiving the approval of the Ethics Committee at the Iraqi Ministry of Health and Environment. The patients attended the outpatient gastrointestinal clinics at Al-Kindy Teaching Hospital, Baghdad Teaching Hospital and Gastroenterology and Hepatology Teaching Hospital in Baghdad for diagnosis and treatment. The diagnosis is made by consultants at the clinics. It is based on standard clinical, radiological, endoscopic and histopathological criteria[16]. Patients with other associated autoimmune diseases are excluded. The control sample included blood donors who are healthy and their serum profile for anti-pathogen antibodies is negative (Central Blood Bank, Baghdad).

Stool sample collection: The sample is collected by using a sterile stool cap that contains (10 ml) of Carry-Blair transport media. Then the sample incubates in BHIB for 24 hr. at 37˚C after that, a loop full of bacterial culture from incubating tubes are streaked separately into the Blood agar, Nutrient agar, MRS agar, MacConkey agar, Yersinia agar, Mannitol salt agar, Ss agar, Chocolate agar, Campylobacter agar, Eosin methylene blue agar and incubating for 24 hrs at 37˚C in aerobic and anaerobic conditions.

Identification of Bacterial isolates: Bacteriaare isolated as single colonies ondifferent selective media including Blood agar, Man-Rogosa- Sharpe agar (MRS), MacConkey agar, Mannitol salt agar, Ss agar, Eosin methylene blue agar, chocolate agar, Yersinia Selective Agar and Campylobacter Base agar that incubate at 37°C for 24 hrs. The primary identification of the bacterial isolateis based on a macroscopical examination that depends on colonial morphology characteristics and microscopical examination via using Grams stain technique, catalase and oxidase test. Confirmation diagnosis of bacterial isolates are using Vitek2 System[17,18].

Detection of virulence Factors:

Biofilm formation:

- **Congo red method:** A single isolate colony is aerobically incubated on congo red agar for (24-48) hrs. at 37 ^{0}C. The high positive result show as the development of black colonies with a dry crystalline and the low positive result show as black colonies without a crystalline dry color, while negative results show as red colonies [19].
- **Microtiter Plate Method:** In the brain heart infusion brothbacterial isolates are inoculated with 1% glucose. Broths are incubating at 37 C for 24 hrs. A (180) µl, brain heart infusion broth, is applied to sterile 96- well polystyrene microtiter plates and loaded with 20 µl of bacteria isolates. Following incubation time, all wells are washed 3 times with 0.2 ml of phosphate buffer saline (PBS) to remove unattached bacteria while remaining biofilms are fixing with 0.1% of crystal violet for 15 minutes after that, the excess stain is wash with PBS. The

Crystal violet attaches to the biofilm has been fixed with 0.2 ml ethanol- acetone mixture (80:20 w/v). The results reading by ELISA reader at a wavelength of 680 nm[20].

Detection of Phospholipase Production: About 5 µl of bacterial inoculum equal to (1.5×10^8 CFU/ml) is inoculated onto the surface of Egg-Yolk agar. Plates incubate at 37C° for 24 hrs., colonial diameter and precipitation zone diameter are calculated (21). The result of phospholipase activity is calculated by using this formula: Pz value= Colony diameter/(Colony diameter + Zone of precipitation). When Pz =1 means the strain is negative, while a value of Pz<0.64- 0.99 = moderate activity and <0.63 = large activity[22].

Detection of Hemolysin Activity: Hemolysin activity is assessed onthe blood agar plate. Bacterial isolates 10µl, equal to (1.5×10^8 CFU/ml) is deposited onto the medium. The plates are then incubated at 37C° for 24 hrs, theratio of the colony's diameter to that of the translucent hemolysis zone (in mm) is used as the hemolytic index (Hz value). The results of hemolytic activity (Hz) were calculated by using this formula: Hz value = Colony diameter/(Colony diameter + Zone of precipitation). The Hz index: Hz < 0.69; large hemolysin activity, Hz = 0.70- 0.79; moderate and the negative activity Hz =1[21].

Antibiotic Sensitivity Test: All isolates are tested for antimicrobial susceptibility following the CLSI (2016) criteria by using the agar diffusion method as follows: few colonies (2-4) from overnight cultivation are transferred to 2 ml of normal saline to prepare the bacterial suspension adjusted to 0.5 McFarland turbidity equivalent to (1.5×10^8 CFU/ml). A sterile cotton swab is used to inoculate the bacterial suspension in Muller Hinton agar plates. Different antimicrobial discs with six discs were used {azithromycin (AZM), tetracycline (TE), ciprofloxacin (CIP), cefixime (CFM), imipenem (IPM) and erythromycin (E)} place on the surface of the medium and the plates are incubating at 37°C for 24 hrs. Measure the diameter of each antibiotic disc's inhibition zone and interpret the results by referring to the CLSI recommendation.

Statistical Analysis: Data were statistically analyzed using the package IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.). Pearson's Chi-squared, Fisher's exact tests and analysis of variance (ANOVA) were used to compare categorical variables and derive significant.

Results and Discussion

Identification of Bacteria: Fifty fecal samples of IBD (UC and CD) patients have been collected and 121 bacterial isolates are isolated on different selective laboratory media. The three most abundant bacterial phyla across all samples are Proteobacteria (54.5%), Actinobacteria (1.6%) and Firmicutes (43.8%). Whereas, at the genus/species level, found the seventeen genera of the bacterial isolate from IBD disease, the bacterial name and number are distributedin table 1.

On the other hand, also fifty samples of stool from apparently healthy people have been collected and 68 bacterial isolates are isolated on different selective laboratory media. At the phylum level, the bacteria isolates are Proteobacteria (49%) and Firmicutes (51%). While in species-level, the name and number of four bacterial isolates are distributed in table 1.

Table 1: Numbers and Percentage of Bacterial Isolates in IBD Patients and healthy control

Bacterial Isolates	IBD isolates No.	Percentage (%)	Control isolates No.	Percentage (%)
Aeromonas salmonicid	1	0.8	0	0.0
Burkholderiacepacia	2	1.6	0	0.0
Citrobacter spp.	11	9	2	2.94
E. coli	28	23.1	31	45.6
Enterobacter aerogenes	2	1.6	0	0.0
Enterobacter cloacae	1	0.8	0	0.0
Enterococcus faecium	4	3.3	0	0.0
Klebsiella pneumonia	6	4.9	0	0.0
Kocuriakristinae	1	0.8	0	0.0

Bacterial Isolates	IBD isolates No.	Percentage (%)	Control isolates No.	Percentage (%)
Lactobacillus acidophilus	20	16.5	10	14.7
Lactobacillus casei	0	0.0	8	11.76
Lactobacillus plantarum	12	9.9	11	16.17
Leuconstocmesenteroidessppcremoris	1	0.8	0	0.0
Micrococcus leteus	1	0.8	0	0.0
Proteus spp.	5	4.1	0	0.0
Pseudomonas putida	1	0.8	0	0.0
Salmonella enterica	6	4.9	0	0.0
Serratia spp.	3	2.4	0	0.0
Staphylococcus aureus	15	12.3	6	8.82
Streptococcus iniae	1	0.8	0	0.0

The microbial variation between Ulcerative colitis and Crohn's Disease: The gut microbial composition variation reveals no significant difference in all bacterial species between UC and CD patients such as summarized in table 2.

Table 2: Bacterial Variation between UC and CD patients

Bacterial Species	UC	CD	Total	P-value
Aeromonas salmonicid	0	1	1	0.488
Burkholderiacepacia	1	1	2	1.000
Citrobacter spp.	5	6	11	0.759
E. coli	15	13	28	0.832
Enterobacter aerogenes	2	0	2	0.496
Enterobacter cloacae	1	0	1	1.000
Enterococcus faecium	2	2	4	1.000
Klebsiella pneumonia	3	3	6	1.000
Kocuriakristinae	1	0	1	0.488
Lactobacillus acidophilus	10	10	20	1.000
Lactobacillus plantarum	6	6	12	1.000
Leuconstocmesenteroides	0	1	1	0.488
Micrococcus leteus	0	1	1	0.488
Proteus spp.	3	2	5	1.000
Pseudomonas putida	1	0	1	0.488
Salmonella enterica	3	3	6	1.000
Serratia spp.	1	2	3	0.613
Staphylococcus aureus	8	6	14	0.778
Staphylococcus cohnii	0	1	1	0.488
Streptococcus iniae	0	1	1	0.488

Several studies document intestinal microbiota of healthy individuals is known to provide the host with multiple health benefits such as diet metabolism and contributing to the immune system by protection against

the pathogen. The intestinal microbiota coevolves with humans and the maintenance of human health by including several symbiotic relationships between the host and the microbiota. An undesirable alteration function and composition of a microbial community called "dysbiosis", that changes the relationship between host−microbiota and the host immune system that demonstrate in the chronic inflammation with IBD patients[10,24,25]. The populations of microbiota imbalance or alteration induce pathogenicity mainly by restricting compounds of beneficial bacteria or by increasing proinflammatory species[26].

A more recent study for microbiome differences among IBD reports the no significant difference link between the distribution of type lesions and bacterial community in IBD patient, although may little diversity in some species according to disease activity[27].

Microbial Variation between IBD Patient (UC and CD) and Control: The microbial isolates alteration between IBD patients and control shown in figures 1. The result showed a clear and significant separation between patients and control, which were the large range of microbial species diversity and altered composition in patients compared to control that have a small microbial species.

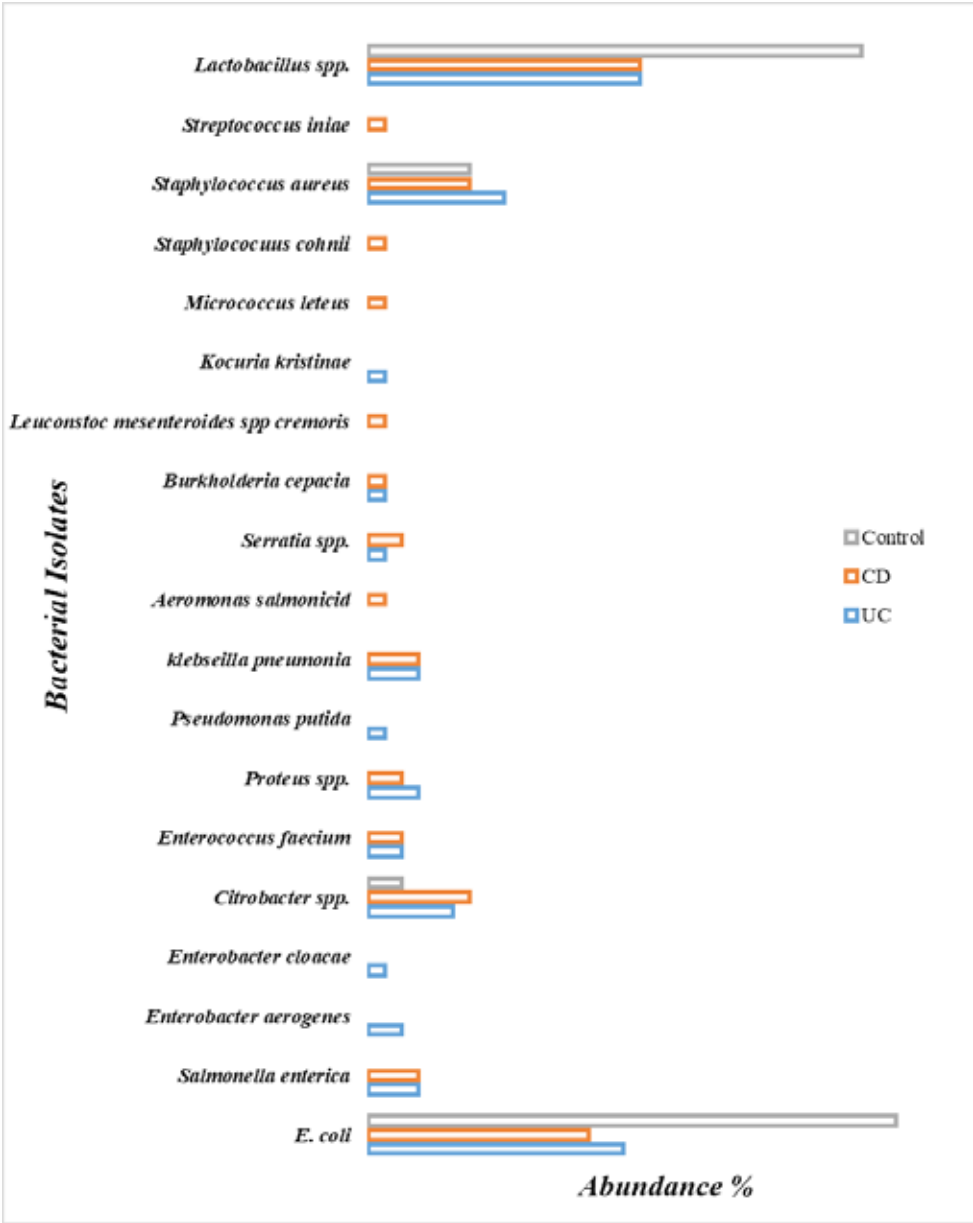

Figure 1: The different bacterial isolates between UC, CD and controls.

These results may fit with other studies that suggest the microbiological profiles are a significant difference between IBD and healthy control[28]. Another study documents the important role of a significantly increasing Proteobacteria, Actinobacteria phylum and decreasing of Firmicutes, Bacteroides and Clostridiaceae phyla in the etiopathogenesis of IBD when compared with control [29]. Inflame samples have several taxa belong to the Proteobacteria phyla show lower transcriptional activity although being present in larger numbers compared to non-inflamed samples[30]. The increase in *Staphylococcus, Enterococcus, Bifidobacterium, Lactobacillus, Klebsiella Pseudomonas, and Proteus* genera, are common bacterial features associate with IBD[31]. Reducing of Firmicutes levels identified among UC and CD patients when compared to healthy individuals [32]. In UC and CD patients increasing of Enterococcus genus when compared with healthy people. These changes in the wall of the intestinal may be resulting inan inflammatory process ongoing, which make it easier for bacteria growing and access nutrients[33].

Virulence Factors:

Biofilm Formation: Congo red method (CRA) and microtiter plate method is used for the detection of biofilm formation. A total of 121 clinical isolates from IBD and 68 isolates from controls are undergone to a method of biofilm detection. The results showed in table 3.

Table 3: The Biofilm results of IBD patients and control on CRA *vs*. microtiter plate method

Groups	Biofilm formation			Total
	High-producer	Moderate- producer	Non-producer	
IBD patients (N= 121)	20 (16.6%) ***vs***. 25 (21%)	86 (71.1%) ***vs.*** 84 (69%)	15 (12.3%) ***vs.*** 12 (10%)	100% (121)
Controls (N= 68)	0 ***vs.*** 1 (1%)	39 (57.3%) ***vs.*** 42 (62%)	29 (42.6%) ***vs***. 25 (37%)	100% (68)

For several recalcitrant infections, biofilm-forming by bacteria is responsible for a highly difficult to eliminate.

They exhibit antibiotic resistance by various method such as restrict antibiotic penetration into biofilms, resistance gene expression and decrease growth rate[34]. Incomplete infection clearance caused by the production of biofilm can result in a chronic infection that led to serious outcomes. For that reason, biofilm detection in such cases is essential to allow choosing a better antimicrobial treatment[35].

The bacteria or substance aggregation are the most important virulent factor that increases in these diseases due to the ability for changes of the intestinal wall. The higher bacterial concentration then relates to more extensive intestinal inflammation and thus to worse disease progression[33]. The increase bacterial number is detected relate to IBD patient's mucosa compared to irritable bowel syndrome patients and healthy control, this might be associate with failure of maintenance of mucosal barrier integrity that resulting in a reduce ability for infection clearance[36].

The results of the CRA method show (16.6% *vs.* 21) high producer organisms, (71.1% *vs.* 69%) moderate producer and (12.3% *vs.* 10%) non-producer bacteria when compared with the microtiter plate method in IBD patient these differences may be an associate with CRA a good method for identifying strong biofilm producers, but it is difficult to distinguish between moderate, weak and non-producer's biofilm according to the variability of the results identify by different observers [20].

Manandhar *et al*., (2018) documented the decreased accuracy of the CRA method in biofilm production, despite being simpler and faster, the CRA approach cannot be relied on for accurate detection of biofilm producers in the routine diagnostic laboratory. The microtiter plate method was the best method among other detection method for detection of biofilm formation by pathogens extracted from our samples because of a strong specificity performance and fewer subjectivity

errors; it can be routinely used for biofilm detection in the microbiology laboratory [35].

Production of Phospholipase Enzyme

Results showed difference ability of 121 IBD isolates and 69 0f control isolates to produce phospholipase enzyme showed in the table 4.

The phospholipase enzyme digests the phospholipid host cell membrane and causes lysis of the cell and surface feature changes that improve adherence, which can be used as one of the parameters to distinguish virulent invasive strains from non-invasive strains and thus result in infection, by reducing a hydrophobic barrier and enable the commensal bacteria to invasion and inducing inflammation [22,38].

This result is related to the positive result of another study that demonstrates the phospholipase enzyme activity increase of fecal intestinal microbiota in the inflammatory stage in UC patients and has a role in the pathogenesis of the disease[38].

Hemolysin Production: The enzyme hemolysin is produced by different clinical isolates from patients infected with IBD out of 121 total isolates and 69 of control isolates as in table 4.

Table 4: The hemolysin and Phospholipase activities results of IBD patients and control

Groups		Number of isolates (%)			Total
		Large activity	Moderate activity	Negative activity	
Hemolysin	IBD patients (N= 121)	42(35%)	49(40%)	30(25%)	121 (100%)
	Controls (N= 68)	17(25%)	24(35%)	27 (40%)	68(100%)
Phospholipase	IBD patients (N= 121)	25(20.5%)	53(44%)	43(35.5%)	121(100%)
	Controls (N= 68)	3(4.5%)	20(29.5%)	45(66%)	68(100%)

Hemolysin enzyme provided a significant role in the pathogenesis of organisms to induced diseases. They aid in the host cell membrane lysis, escape the immune system and the nutrients released for the microbe's survival and development of disease[39].

Antimicrobial Susceptibility Assay: The results of the antibiotic sensitivity test show relied on measuring the diameter of inhibition zone and comparing it with CLSI (2016) (Clinical & Laboratory Standards Institute) the gram-negative bacteria of patients (*K.pneumonia*e, *Enterobacter* spp., *S. enterica, E. coli, Proteus spp., P. putida, Burkholderiacepacia, Serratia* spp*., A. salmonicid* and *Citrobacter* spp.) display a different response for five antibiotics in figure 2. Out of 66 gram-negative isolates, the antibiotic imipenem was the most effective antibiotic against gram-negative bacteriain thesensitive proportion (76%) while the resistance of this antibiotic (24%), The proportion of resistance to azithromycin (85%), tetracycline (61%) and ciprofloxacin (71%) while the lower effective are in cefixime that (88%) of the resistance proportion.

Whereas out of 55 gram-positive isolates of patients (*Streptococcus iniae, Staphylococcus* spp*, M. leteus, K. Kristinae, Leuconstocmesenteroid, E. faecium,* and *Lactobacillus* spp.) display a different response for five antibiotics in figure 2, where the antibiotic imipenem is the most effective antibiotic against gram-positive bacteria with the resistance proportion (27%) and the sensitive proportion (73%), whereas the proportion of resistance to azithromycin is (80%), erythromycin (91%) and ciprofloxacin (73%) while the lower effective are in cefixime that (95%) of the resistance proportion.

On the other hand, the out of 68-gram negative and gram positive isolates from control (*E. coli, Citrobacter freundii, S. aureus* and *Lactobacillus* spp.) display a different response for five antibiotics in figure 2, where the antibiotic imipenem isa most effective antibiotic against bacteria with the resistance proportion (15%) and the sensitive proportion (85%), whereas the proportion of resistance to azithromycin (37%), cefixime (26%) and ciprofloxacin (28%), while the lower effective for gram-positive isolates the resistance proportion of erythromycin is (43%)and gram-negative isolates the tetracycline (45%).

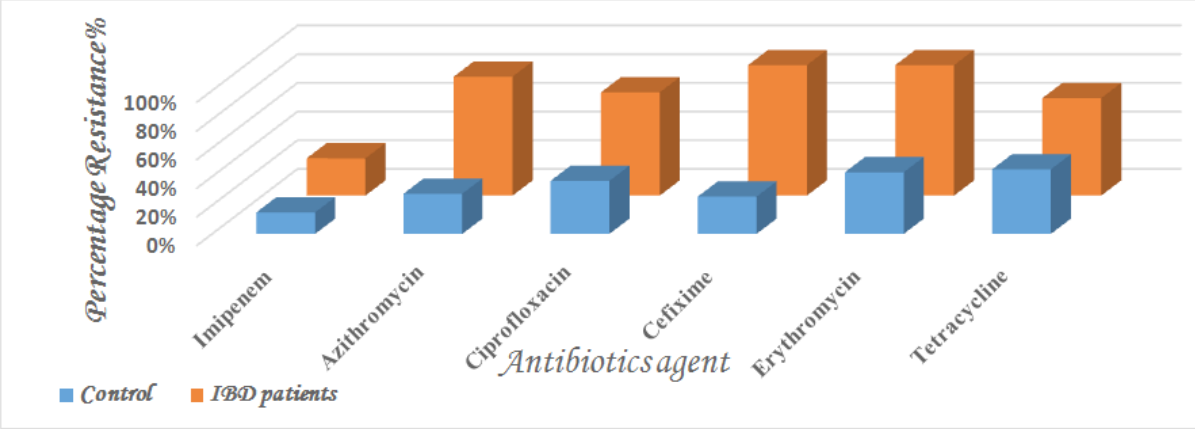

Figure 2: The proportion of antibiotic resistance of IBD patients and control toward six antibiotics

Resistance to antibiotics are commonly understood to be associate with genetic modifications or achieve without any genetic modification, called non-inherited resistance phenotypic is correlate with certain conditions, such as biofilm formation, a stationary phase of growth[40]. Bacteria in biofilms form exhibit higher antibiotic tolerance than planktonic form, so MIC value for bacterial biofilms can be up to 1000–1500 times higher than those with planktonic bacteria[20].

In this study we found a significant difference between IBD patient and control among antibiotics sensitivity, in my opinion maybe this a reflection of the association disease, alteration in microbiota interaction, diet, immunosuppresses drugs (infliximab) and other antibiotics that may give resistance to some drugs, biofilm and other virulence factors.

Conclusion

In the present study, we can conclude that microbiota alteration in IBD patients (both CD and UC) and healthy volunteers have an imbalance in microbial communities that have different phylum and species levels relative to healthy volunteers and in different activity in their virulence factors.

Acknowledgment: The authors would like to thank Mustansiriyah University, Iraq (www.uomustansiriyah.edu.iq) for its support of the current work.

Source of Funding: Self fund.

Conflict of Interest: No conflict of interest

Ethic Statement: The researchers already have ethical clearance from all required institution and laboratories.

References

1. Burisch J, Munkholm P. The epidemiology of inflammatory bowel disease. Scandinavian Journal of Gastroenterology. 2015; 50: 942-951.
2. Bernstein C, Eliakim A, Fedail S, Fried M, Gearry R, et al. World Gastroenterology Organisation Global Guidelines Inflammatory Bowel Disease. Journal of Clinical Gastroenterology. 2016; 50(10): 803-818.
3. Kuhnen A. Genetic and environmental considerations for inflammatory bowel disease. Surgical Clinics of North America. 2019; 99:1197–1207.
4. Haque S, Haque M. The ecological community of commensal, symbiotic, and pathogenic gastrointestinal microorganisms -an appraisal. Clinical and Experimental Gastroenterology. 2017; 10: 91–103.
5. Pickard J, Zeng M, Caruso R, Núñez G. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. Immunological reviews. 2017; 279(1): 70–89.
6. Bäckhed F, Ley R, Sonnenburg J, Peterson D, Gordon J. Host-bacterial mutualism in the human intestine. Science. 2005; 307(5717): 1915-1920.
7. Cho I, Yamanishi S, Cox L, Methé B, Zavadil J, et al. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature. 2012; 488: 621–626.
8. Singh P, Teal T, Marsh T, Tiedje J, Mosci R, et al. Intestinal microbial communities associated with acute enteric infections and disease recovery. Microbiome. 2015; 3(1): 1-12.

9. Zhang M, Yang, X. Effects of a high fat diet on intestinal microbiota and gastrointestinal diseases. World Journal of Gastroenterology. 2016; 22(40): 8905.
10. Nishida A, Inoue R, Inatomi O, Bamba S, Naito Y, Andoh A. Gut microbiota in the pathogenesis of inflammatory bowel disease. Clinical journal of gastroenterology. 2018; 11(1): 1-10.
11. Rodiño-Janeiro B, Vicario M, Alonso-Cotoner C, Pascua-García R, Santos J. A review of microbiota and irritable bowel syndrome: future in therapies. Advances in Therapy. 2018; 35(3): 289-310.
12. Zuo T, Ng S. The gut microbiota in the pathogenesis and therapeutics of inflammatory bowel disease. Frontiers in microbiology. 2018; 9: 2247.
13. Schäffler H, Herlemann D, Alberts C, Kaschitzki A, Bodammer P, et al. Mucosa attached bacterial community in Crohn's disease coheres with the clinical disease activity index. Environmental microbiology reports. 2016; 8(5): 614-621.
14. Halfvarson J, Brislawn C, Lamendella R, Vázquez-Baeza Y, Walters W, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nature Microbiology. 2017; 2(5): 1-7.
15. Dong L, Wang M, Guo J, Wang J. Role of intestinal microbiota and metabolites in inflammatory bowel disease. Chinese Medical Journal. 2019; 132(13): 1610-1614.
16. Flynn S, Eisenstein S. Inflammatory bowel disease presentation and diagnosis. Surgical Clinics of North America. 2019; 99: 1051–1062.
17. Forbes B, Daniel F, Alice S. Diagnostic microbiology. 12th. ed., 2007, Mosby Elsevier Company, USA.
18. Brown A. Benson's Microbiological Application .9th ·ed 2005. The McGraw-Hill Companies, USA. Science.
19. Bazargani M, Rohloff J. Antibiofilm activity of essential oils and plant extracts against Staphylococcus aureus and Escherichia coli biofilms. Food Control. 2016; 61: 156-164.
20. Bhardwaj A, Kharkwal C, Singh A. A Comparative Appraisal of Detection of Biofilm Production Caused by Uropathogenic Escherichia coli in Tropical Catheterized Patients by Three Different Method. Asian Journal of Pharmaceutics. 2018; 12(04): S1445-S1450.
21. Tsang C, Chu F, Leung W, Jin L, Samaranayake L, Siu S. Phospholipase, proteinase and hemolytic activities of Candida albicans isolated from oral cavities of patients with type 2 diabetes mellitus. Journal of Medical Microbiology. 2007; 56(10): 1393-1398.
22. Deepa K, Jeevitha T, Michael A. In vitro evaluation of virulence factors of Candida species isolated from oral cavity. Journal of Microbiology and Antimicrobials. 2015; 7(3): 28-32.
23. CLSI, (Clinical and Laboratory Standards Institute). Performance standards for antimicrobial susceptibility testing. Twenty-six informational supplement. 2016; 36(1): M100-S26.
24. Matijašić M, Meštrović T, Perić M, Čipčić-Paljetak H, et al. Modulating Composition and Metabolic Activity of the Gut Microbiota in IBD Patients. International Journal Of Molecular Sciences. 2016; 17(4): 578. doi: 10.3390/ijms17040578
25. Glassner K, Abraham B, Quigley E. The microbiome and inflammatory bowel disease. Journal of Allergy and Clinical Immunology. 2020; 145(1): 16-27.
26. Zhou M, He J, Shen Y, Zhang C, Wang J, Chen Y. New Frontiers in Genetics, Gut Microbiota, and Immunity: A Rosetta Stone for the Pathogenesis of Inflammatory Bowel Disease. Biomed Research International. 2017; 2017: 1-17. https://doi.org/10.1155/2017/8201672
27. Stange E, Schroeder B. Microbiota and mucosal defense in IBD: an update. Expert Review of Gastroenterology and Hepatology. 2019; 13(10): 963-976.
28. Santoru M, Piras C, Murgia A, Palmas V, et al. Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. Scientific Reports. 2017; 7(1): 9523. doi: 10.1038/s41598-017-10034-5.
29. Rosso A, Aguilera P, Quesada S, Cerezo J, et al. New insights in Ulcerative Colitis Associated Gut Microbiota in South American Population: Akkermansia and Collinsella, two distinctive genera found in Argentine subjects. MedRxiv. 2020. doi: https://doi.org/10.1101/2020.07.29.20164764
30. Moen A, Lindstrøm J, Tannæs T, Vatn S, et al. The prevalence and transcriptional activity of the mucosal microbiota of ulcerative colitis patients. Scientific Reports. 2018; 8(1): 17278. doi: 10.1038/s41598-018-35243-4.

31. Salem F, Kindt N, Marchesi J, Netter P, et al. Gut microbiome in chronic rheumatic and inflammatory bowel diseases: Similarities and differences. United European Gastroenterology Journal. 2019; 7(8): 1008-1032.
32. Younis N, Zarif R, Mahfouz R. Inflammatory bowel disease: between genetics and microbiota. Molecular Biology Reports. 2020; 47(4): 3053-3063.
33. Růžičková M, Vítězová M, Kushkevych I. The characterization of Enterococcus genus: resistance mechanisms and inflammatory bowel disease. Open Medicine. 2020; 15(1): 211-224.
34. Hassan A, Usman J, Kaleem F, Omair M, et al. Evaluation of different detection method of biofilm formation in the clinical isolates. Brazilian Journal Of Infectious Diseases. 2011; 15(4): 305-311.
35. Sultan A, Nabiel Y. Tube method and Congo red agar versus tissue culture plate method for detection of biofilm production by uropathogens isolated from midstream urine: Which one could be better?. African Journal of Clinical and Experimental Microbiology. 2019; 20(1): 60-66.
36. Vestby L, Grønseth T, Simm R, Nesse L. Bacterial Biofilm and its Role in the Pathogenesis of Disease. Antibiotics. 2020; 9(2): 59. doi: 10.3390/antibiotics9020059.
37. Manandhar S, Singh A, Varma A, Pandey S, Shrivastava N. Evaluation of method to detect in vitro biofilm formation by staphylococcal clinical isolates. BMC Research Notes. 2018; 11(1): 714. doi: 10.1186/s13104-018-3820-9.
38. Stremmel W, Staffer S, Stuhrmann N, Gan-Schreier H, et al. Phospholipase A2 of Microbiota as Pathogenetic Determinant to Induce Inflammatory States in Ulcerative Colitis: Therapeutic Implications of Phospholipase A2 Inhibitors. Inflammatory Intestinal Diseases. 2017; 2(3): 180-187.
39. Divyakolu S, Chikkala R, Ratnakar K, Sritharan V. Hemolysins of Staphylococcus aureus— An Update on Their Biology, Role in Pathogenesis and as Targets for Anti-Virulence Therapy. Advances In Infectious Diseases. 2019; 09(02): 80-104.
40. Fernando C. Jose L. Phenotypic resistance to Antibiotics. Antibiotics. 2013; 2: 237-255.