Implication of Gut Microbiome in Pathophysiology of Irritable Bowel Syndrome (IBS)

By

ZUMARA YOUNUS

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad Pakistan

2022

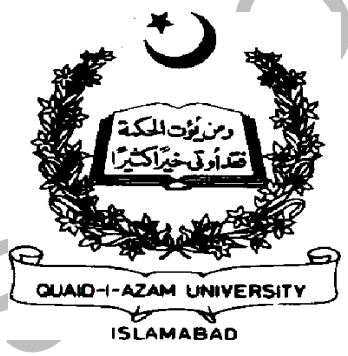
Implication of Gut Microbiome in Pathophysiology of Irritable Bowel Syndrome (IBS)

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Microbiology



By

ZUMARA YOUNUS

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad Pakistan

2022

In the name of Allah,

the most beneficent, the most merciful

DEDICATED

TO

My Beloving Husband

Adeel Ahmed Tariq

For his advice, his patience

And love of my life my

daughters

Manha Fatima

And

Ayzel fatima

DECLARATION

The material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Zumara Younus

Author's Declaration

I **Ms. Zumara Younus** hereby state that my Ph.D. thesis titled **"Implication of Gut Microbiome in Pathophysiology of Irritable Bowel Syndrome (IBS)"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my Ph.D. degree.

Ms. Zumara Younus
Date: 18-08-2022

Plagiarism Undertaking

"Implication of Gut Microbiome in Pathophysiology of Irritable Bowel Syndrome (IBS)" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature: _____

Name: **Ms. Zumara Younus**

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Implication of Gut Microbiome in Pathophysiology of irritable Bowel Syndrome (IBS)"** was conducted by **Ms. Zumara Younus** under the supervision of **Dr. Aamer Ikram, SI (M) and Co-Supervision of Dr. Muhammad Imran.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

Student Name: **Ms. Zumara Younus** Signature:_____

Examination Committee:

a) External Examiner 1: Signature:_____

Prof. Dr. Azra Khanum
University Institute of Biochemistry & Biotechnology
PMAS Arid Agriculture University, Murree Road
Rawalpindi

b) External Examiner 2: Signature:_____

Dr. Shahzad Hussain
House No. 1082, Street No 7,
OverseaseBlock-6 Bahria Town, Phase-8, Rawalpindi

Supervisor Name: **Dr. Aamer Ikram, SI (M)** Signature:_____

Co-Supervisor Name: **Dr. Muhammad Imran** Signature:_____

Name of HOD: **Prof. Dr. Aamer Ali Shah** Signature:_____

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Abbreviations	Description
IBS	Irritable bowel syndrome
IBD	Irritable bowel disease
Rrna	Ribosomal ribonucleic acid
SCFAs	Short chain fatty acids
GIT	Gastrointestinal track
UC	Ulcerative colitis
CD	Crohn's disease
RHS	Reflux hypersensitivity syndrome
CHS	Cannabinoid hyperemesis syndrome
OIC	Opiod-induced constipation
NBS	Nacrotic bowel syndrome
IBS-C	Irritable bowel syndrome constipation
IBS-D	Irritable bowel syndrome diarrhea
IBS-M	Irritable bowel syndrome mixed
HAPCs	high-amplitude propagated contractions
PI-IBS	Post infectious Irritable bowel syndrome
TJP	Tight junctions protein
LPS	Lipopolysaccharide
TLR-4	Toll like receptor 4
NF-KB	Nuclear factor kappa b
MAPK	Mitogen-activated protein kinase
ZO-1	Zonula occludens-1
PGC-1	Proliferator-activated receptor
LXR	Liver X receptor

FXR	Farnesoid X receptor
UCP	Uncoupling protein
HSL	Hormone-sensitive lipase
IGN	Intestinal gluconeogenesis
GLP-1	Glucagon-like receptor
PCR	Polymerase chain reaction
FGIDs	Functional gastrointestinal disorders
SIBO	Small intestinal bacterial overgrowth
NAFLD	Non alcoholic fatty liver disease
ALT	Alanine transaminase
AST	Aspartate transaminase
ALP	Alkaline phosphatase
GGT	Gamma-glutamyl transpeptidase
CBC	Complete blood count
WBC	White blood cells
RBC	Red blood cells
HDL	High density lipoprotein
LDL	Low density lipoprotein
VLDL	Very low density lipoprotein
Anti-TTG	Anti- Transglutaminase
GC-FID	Gas-chromatography Flame ionization detector
Ac	Acrylic acid
ANC	Absolute neutrophil count
GC-MS	Gas chromatography mass spectrophotometry
TRFLP	Terminal restriction fragment length polymorphism
DGGE	Denaturing gradient gel electrophoresis
QIIME	Quantitative insights into microbial ecology

TBE	Tris/Borate/EDTA
PCoA	Principal coordinate analysis
NGS	Next genome sequencing
OUT	Operational taxonomical units
MAC	MacConkey agar
UMGC	University of Minnesota Genomic center
NCBI	National center for bioinformatics
CFU	Colony forming unit
PGDB	Pathway genomic data base
CARD	Comparative antibiotic resistance database
AAC	Aminoglycoside acetyl transferase
AMR	Antimicrobial resistance gene
PMQR	Plasmid mediated quinolone resistance genes
GC	Guanine cytosine
RND	Resistance nodulation cell division
MFS	Major facilitator superfamily
CD	Coding sequences

Acknowledgements

Foremost I want to offer this endeavor to our **Almighty Allah,** who deserves all the thanks and obedience in its best possible way for the wisdom he bestowed upon me, the strength, peace of mind and good health in order to finish this research. Peace upon the **Prophet Muhammad (PBUH),** who is one of the best sources of knowledge and guidance for humanity.

I owe my thanks to my supervisor, **Prof Maj. Gen. Dr. Aamer Ikram** for his kind supervision, it is a great honor to work under his supervision. Due to his encouragement, creative and comprehensive advices I am able to do my project. I am truly grateful and forever indebted **Dr. Muhammad Imran,** my Co-supervisor for his continuous support during my PhD study and research, for his patience, enthusiasm, and immense knowledge. His guidance helps me all time in research and writing. I could not have imagine having a better advisor and mentor for my PhD study.

My sincere thanks go to Prof Dr Sagar Goyal, for accepting me as visiting scholar at UMN(University of Minnesota) and giving opportunities to work on diverse exciting projects. I would also like to thank the **Faculty of Department of Microbiology-QAU,** for enabling me to build strong theoretical background, and **HEC** for providing me financial support that helped in successful completion of my research work.

I also want to express my whole-hearted thankfulness to my lab fellow's **Dr Nader, Dr Tamer**, **Nadeem, Mediha, Misbah** and **Fillza**. AFIP(Armed Forces Institute Of Pathology) and gastroenteritis department(MH) faculty member have there special role in completing my research work in Pakistan especially **Gen. Dr. Farrukh saeed, Maj Dr. Eshtisham** and **Brig Dr. Ghoar zaman.**

Finally, I would like to appreciate the selfless support of my parents **Muhammad Younus khan** and **Robina kouser** and my lovely brothers **Mahad, Haseeb, Talha**. I want to thank you from core of my heart to all my **In-laws** especially my **Mother In-law** and **Father In-law** for their love and support, this whole journey was possible due to their continuous support. Last and most important all thanks to my big supporter my **Husband Eng. Adeel Ahmed Traiq** and my fairy **Manha fatima** for the compromises and sacrifices they did for my success.

Zumara younus

Abstract

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder, and highly prevalent among general public but its etiology is still unknow. A complex interaction between lifestyle, diet and intestinal microbiome play a role in inception and progression of IBS. Moreover, it is now clear that microbial factors play a key role in IBS pathophysiology. Acute gastric infection along with abnormal gut microbiota leads to IBS condition and studies proved changes in the gut microbiome in IBS patients.

This study was designed to check epidemiological, hematological, and microbial diversity in IBS patients. For study of microbiome in IBS we calculated number of samples using WHO formula (http://www.who.int/chp/steps/resources/sampling/en/), which comes out to be 22, we choose 30 IBS patients to conduct our study. In the first phase of the study, role of anthropometrics parameters on progression of IBS was studied along with serum biochemistry analysis of IBS patients. Results concluded that IBS is disease of all ages having more prevalence in males then females (4:1). Diet particularly don't have any vigorous effect in elevating IBS symptoms while reducing physical activity can elevate symptoms severity. IBS have three subtypes based upon symptoms i.e IBS-C (constipation),IBS-D (diarrhea) and IBS-M (mixed). In our study 56% of patients had IBS-Mixed symptoms while IBS-C and IBS-D reported to be 17 and 27% respectively. One of the important factors noted in triggering IBS was stress level, 50% of patients were having high-stress level when evaluated using a questionnaire. It was also noted that IBS symptoms were periodic and reoccur after certain time. IBS patients usually have long history of the disease, from our data we come to know that 27 % of patients were having symptoms from the last five years while 14 % had symptoms for more than half of their life.

No drastic changes in hematological parameters of IBS patients were seen, a negative correlation was seen between IBS and vitamin D level, while that of liver enzymes, lipid profile, and complete blood picture remain the same in almost all patients. SCFAs are fermented by-products of bacterial metabolism, one of the important is butyrate production. As in IBS butyrate-producing, the bacterial level decreases so in general production of butyrate decrease in IBS patients.

The second phase of study was gut microbiota changes in IBS patients using high

throughput sequencing technology. As fecal microbiota is representative of intestinal microflora so fecal sample was used to study intestinal dysbiosis in IBS patients. Five high prevalent bacterial phyla in IBS patients were: Firmicutes(94.9%), followed by Actinobacter, Bacteroidetes, Proteobacteria, and Tenericutes. Most of common genus present were *ruminococcus and lactobacillus* while that of *Bifidobacterium* and *faecalibacterium* were low. Another important order found was clostridia and bacilli which had an association of increasing gut dysbiosis, while those related to SCFAs production like Ruminococcaceae family were present in lower numbers. In the Family Enterobacteriaceae species of shigella/ salmonella were observed which showed association of diarrheal like symptoms in IBS patients. Gastroenteritis causing genus like Aeromonas, Weissella and Paenibacillus also increase in IBS patients.

Comparing different IBS subtypes on basis of bacterial diversity we observe that IBS-M have higher number of bacteriodetes and cynobacteria, while IBS-D have firmicutes and tenricutes. Fusobacteria was observed in higher number in IBS-C. Depression also changes gut microbial diversity among IBS patients, interesting interactions were found between many bacterial species and patients having high depression level, most prevalent phylum present in high level of depression patients was Enterobacteriaceae, Firmicutes phylum was also high in IBS patients having high level of depression and among this phyla genus Weissella, bromii, belonging to family Leuconostocaceae and Ruminococcaceae respectively ratio was more.

Third phase was study of pathogenesis of culturable microbiota from IBS patients using long read sequencing approach. Main objective of study was to look into genetic diversity of culturable microorganism. Culturing, sanger sequencing and MinION techniques were used in this phase which revealed presence of important gram positive (Enterococcus) and pathogenic gram-negative species in IBS patients' stool. One of important pathogen found was *Klebsiella pneumonia,* although *K. pneumonia*, present in normal healthy helps them to digest carbohydrates such as lactose, resistant starches, inulin, fructose, and mannose but this can cause infection in intestine when it became opportunistic and increase in numbers. *E. coli* which is mostly associated with travelers' diarrhea and gut dysbiosis, its Strains were enriched in virulence genes associated with extraintestinal pathogenic *E. coli* (ExPEC) and/or

adherent-invasive *E. coli* are related to IBS symptoms like dysbiosis were also found in our samples. *Shigella flexneri* which, altered bowel motility, visceral hypersensitivity, psychosocial distress, abnormal brain-gut interaction, enteric infection, gut-immune activation, low grade inflammation, intestinal permeability, and alterations in intestinal microflora were also present among IBS patients. Few strains of *Proteus mirabilis* causing bloating, abdominal discomfort and changes in stool form among IBS patients were also studied for their genetic diversity and virulence factor presence. *Enterococcus* now termed as an Emerging Pathogens were also in high abundant among IBS patients. Enterococcus ($p=0.001$) exhibited higher levels in IBS patients. Virulence and pathogenicity factors such as adhesins, invasions, pili, and hemolysin in *Enterococcus* an make them human pathogen, we found genes related to these in our *Enterococcus* isolates. In total we check 17 strains for whole genome all have varying amount of resistance genes.

Concluding all results, we can summarize as 50% of IBS patients have symptoms of disease before they reach 35 years, and as age increases to 50 years or above IBS prevalence decreases to 50%. Highest rate of IBS was observed in age group between 31 years-40 years, and the lowest was between 71-80 years of age group. Duration of IBS was the time period patient is suffering from this disease, highest number of patients were recorded between age groups 1-5 i.e. 27. IBS can lead to reducing quality of life(QoL) while xercise can improve feelings and symptoms of fatigue, bloating and constipation.

In our research we found that there was a relative high abundance of proinflammatory bacterial species like Enterobacteriaceae, while a reduced number of lactobacillus and bifidobacterium was observed. Moreover, Bifidobacterium families, all short chain fatty acids (SCFAs) producers were also observed to be lower in IBS patients due to which we observed low concentration of butyrate in IBS patients blood serum. Increased level of streptococcus species were present in IBS patients which shows assosiaction with inflammation . Veillonella in IBS patients and those with higher organic acid levels presented with worse gastrointestinal symptoms, quality of life, and negative emotions(depression). Although we could not successfully assemble the

genomes from metagenomic data at this stage,

However, these results are showing very good diversity at species level. Although culturing reveals that gram negatives were present in all patients, but their quantity was low using culture independent techniques. Most of strains were resistance to common antibiotics like enterococcus strains were mostly resistance to vancomycin. All these resistance strains in IBS patients is an alarming situation for clinicians as they need to treat patients with narrow spectrum antibiotics instead of prescribing directly broad spectrum antibiotics.

.

Chapter 1: Introduction

Irritable bowel syndrome (IBS) is a disease of a modern world characterized by symptoms of inflammation and abdominal problems such as cramps, distress, flatulence, and change in the pattern of bowel movement (either diarrhea, constipation or both)(Shah, Ramos-Garcia, Bhavsar, & Lehrer, 2019). IBS is different from IBD (inflammatory bowel disease) that is commonly used for ulcerative colitis and Crohn's disease.

Literature shows that the earliest case of IBS was first recognized by a physician in the last decades of the 19th century (Cremonini & Talley, 2005). In 2016, Rome IV criteria was developed for the diagnosis of IBS on the basis of symptoms (Drossman, 2016). Rome IV criteria is standard diagnostic guideline for IBS, which depends on intensity and time interval of symptoms .There are many key-players that contribute to the development of IBS such as age, genetic factors, gender and socio-economic condition of the patients. Pathophysiology of IBS is very complex and approaches to look into it have changed over time (Canavan, West, & Card, 2014). The classical approach focuses on colonic dysmotility, visceral hypersensitivity, and the brain-gut interaction while modern approach focus on inflammation, post-infection low-grade inflammation, immunological and genetic factors, dietary habits, dysbiosis in gut microbiota, and default in enteroendocrine cells (Lee & Park, 2014).

At old times, physician-diagnosed IBS after excluding malignancies, inflammatory indicators and undergoing invasive surgeries (Choung, 2011; Khan & Chang, 2010). Nowadays, it is clinically diagnosed on the basis of Rome criteria IV which is an evolution of Rome I, II and III. IBS is 10-15% prevalent worldwide(Grundmann & Yoon, 2010), (Devanarayana *et al*., 2015). While among general Pakistani population, a paper published in 2007 presented that 45% of 1084 participants in Bahawalpur and Karachi were diagnosed by IBS (Ibrahim *et al*., 2016). People usually diagnosed with IBS are in there late teens or above the 40s. Based on the gender of the patient, females are more likely to get diagnosed with IBS(Chang & Lu, 2011).

More than 1014 microbial genera inhabited human gut making it a complex ecosystem, modern techniques like metagenomic analysis revealed that population of gut microbiota is dominant in human gut, gut contains 150 folds more genes of microbes then of human gene. (Lovell & Ford, 2012). In healthy subjects gut microbiota plays several roles like absorption of nutrients from food, in immunity, as they facilitate normal immune responses, another important function performed by gut microbiota is maintaining homeostasis inside living body, keeping in view all these functions performed by microbiota inside body scientist now consider gut microbiota as an virtual organ of body (Distrutti, Monaldi, Ricci, & Fiorucci, 2016),and it's now proved any dis balance in gut microbiota leads to multiple diseases. So it's important to investigate and study gut microbiota, because traditional culture based methods doesn't give us a true picture, as it only able to identify

about 0.1% of microbes living inside human microbe(Hiergeist, Gläsner, Reischl, & Gessner, 2015).

Now new molecular techniques have been developed which help scientist to have more clear picture of gut microbiota. One of most popular technique used now a days is microbiota profiling using 16S ribosomal RNA (rRNA)-this technique basically amplifies hyper variable regions of rRNA genes, and uses know sequences in database to analyze microbial composition(Bhattarai, Muniz Pedrogo, & Kashyap, 2016).Due to its precision and high competence, the 16S rRNA-targeted sequencing technique is more popular and one of most widely used technique for studying of gut microbiota (Mancabelli *et al*., 2017).These molecular assay target specific genomic regions of pathogens but for clinical point of view researchers are now prefer real time metagenomic more comprehensive techniques like oxford nano pore which helps in rapid identification of not only co-infections but also antibiotic resistance genes and molecular adaptations (Kafetzopoulou *et al*., 2019).

Various studies have conducted on gut microbiota shows that gut microbiota changes in patients with IBS. Although gut microbiota of IBS is difficult to understand due to less data but some inferences can be drawn from previous studies i.e. in IBS patient's ratio of firmicutes to Bacteroidetes changes, firmicutes level increases and Bacteroidetes level decreases(Mazzawi, Hausken, Gundersen, & El-Salhy, 2015). Both these phyla are very important and abundant in human gut, as Bacteroidetes is related to production of SCFA butyrate(Bennet, Öhman, & Simrén, 2015).

Butyrate helps in reducing inflammation and marinating normal gut function while firmicutes is related to energy production in gut and they have role in obesity and diabetes(Mariat *et al*., 2009). So firmicutes and Bacteroidetes ratio balance is important in maintaining normal gut functioning but studies now proved 2-fold higher ratio of firmicutes in IBS patients(Rajilić–Stojanović *et al*., 2011). But these studies are not enough to draw evidences, how gut changes are associated with IBS, some scientist proposed that high F:M in obese person can be due to capacity of firmicutes to extract core energy as well as these produces low grade inflammation. (Clemente, Ursell, Parfrey, & Knight, 2012). One of the possible mechanism is changing in epithelial permeability which is one of important components in progression of diseases(Defrees & Bailey, 2017).

An important function performed by anaerobic bacteria of intestinal lumen is production of short chain fatty acid (SCFAs), by fermentation of carbohydrates.(Tan *et al*., 2014). SCFAs have anti-inflammatory and immune modulatory functions as well as act as preserving agents in gut barrier functions as they are end products of fermentation in lumen.(Ahmed, Greenwood, de Lacy Costello, Ratcliffe, & Probert, 2013). Most prevalent SCFAs present in human gut is butyric, acetic and propionic acids, covering 83% of all the SCFAs present in colon. (Velasquez-Manoff, 2015). Substrate for butyrate production is well balanced diet rich in fibers, probiotics and prebiotics,

body produces butyric acids by bacterial fermentation of non-digestible carbohydrates (hexose oligomers non-starch polysaccharides, resistant starch, oligosaccharides (inulin and oligofructose), disaccharides (lactose) and sugar alcohols (sorbitol and mannitol), (Altobelli, Del Negro, Angeletti, & Latella, 2017). Undigested starch is also an important substrate for butyric production.

Clostridium spp, Eubacterium spp, Fusobacterium spp, Butyrivibrio spp., Megasphaera elsdenii, Mitsuokella multiacida, Roseburia intestinalis, Faecalibacterium prausnitzii and Eubacterium hallii are few identified bacteria that have role in production of SCFAs(Farup, Rudi, & Hestad, 2016). Changing in normal gut microbiota leads to decreases in production of SCFAs so measuring SCFAs provides a foundation and characteristic target to analyze intestinal health. Scientist have now seen SCFAs abnormalities in IBS patients, verifying the fact that alterations in SCFAs might be related to IBS although many studies publicized association of IBS and SCFAs but more studies are needed to clarify relationship between SCFAs ratio present in gut and an impaired microbiota of IBS patients (Ringel-Kulka *et al*., 2015).

Vitamin D deficiency is a very common worldwide and know scientist are linking this deficiency to effect human intestinal microbiome, which can lead toward many metabolic disorders like obesity, hypertension, high cholesterol, autoimmune disorders and even to some heart diseases (Frosali *et al*., 2015). Although the role of vitamin D deficiency in IBS has not yet been cleared, studies are going side by side to develop a relationship between IBS progression and vitamin D deficiency. Most of vitamin D (70-80%) is absorbed in ileum, because ileum has most of vitamin D receptor, reduced microbial diversity in ileum can also leads to reduce absorption of vitamin D(Holick, 2009). Treatment of IBS with vitamin D supplements produces very good results as it helps in decreasing anxiety and depression in patients(Atkinson, Sheldon, Shaath, & Whorwell, 2004).Lipid profiling of IBS patients can also be affected because microbial activities in gut are responsible for production of large number of compounds.

1.1. **Objectives of the study**

- To evaluate the Irritable Bowel Syndrome (IBS) pathophysiology of selected patients.
- To determine the role of gut microbiome diversity and its correlations with atmospheric parameters in onset of IBS.
- To understand the potential pathogenesis of culturable microbial strains in IBS patients.

Chapter 2: Review of Literature

The term Irritable Bowel Syndrome (IBS) is commonly used to describe non-inflammatory, large colon disorders characterized by abdominal distress, inflation of lower abdomen, cramps, and change in bowel movement patterns (diarrhea or constipation or both)(Shah, Ramos-Garcia *et al*. 2019). The IBS is easily confused with irritable bowel diseases (IBD), which include inflammatory diseases such as ulcerative colitis (UC) and Crohn's disease (CD). The IBS was first documented at the end of the 19th century and at the start of the 20th century (Cremonini and Talley, 2005). At that time, physicians diagnosed IBS after excluding malignancies, inflammatory indicators and undergoing invasive surgeries. Nowadays, it is clinically diagnosed on the basis of Rome criteria IV which is an evolution of Rome I, II and III (Drossman and Corazziari, 2000).

2.1. Epidemiology of Irritable Bowel Syndrome

At the start of the last century, no definite criteria were available for the diagnosis of IBS, which led to misdiagnosis of this syndrome. In addition, the physicians were unable to ascertain reported cases, which led to false estimation of IBS epidemiology worldwide. However, a small number of patients admitted to hospitals were accurately diagnosed with IBS (Cremonini and Talley, 2009). As mentioned before, there is a lack of biomarkers that can be used for the diagnosis of IBS, which creates hindrances in the study of prevalence of IBS (Thompson *et al.,* 2000). Prevalence studies in a particular community greatly depend upon the general mindset of the papulation related to seeking medical help and tools used for data collection (questioners, telephone calls, and mails etc.). Worldwide prevalence of IBS is 11% (Canavan *et al.,* 2014). Most of the prevalence studies have been carried out in America, Southeast Asia, and Europe. Meta-analysis showed that Southeast Asia has the lowest prevalence of 7% while South America has highest at 21% (Canavan *et al.,* 2014).

2.2. Susceptibility to Irritable Bowel Syndrome

The IBS is a gastrointestinal problem that mainly affects the distal part of large colon. It is, therefore, assumed that the prevalence of IBS is independent of environmental factors. However, it has been shown that multiple factors play a role in the development of IBS, e.g., sex, age, and socioeconomic factors (Canavan *et al.,* 2014).

On the basis of sex, many studies have shown that the prevalence of IBS is 1.5- to 3-fold higher in females than in males. However, in Asia, South America, and Africa, the prevalence in the male population is nearly equal to that in females and often higher (Canavan *et al.,* 2014). One reason for this ambiguity could be that females are more common visitors to the physician (Canavan *et al.,* 2014). On the basis of age, 50% persons under the age of 35 years are diagnosed with IBS while only 25% of cases are in adults over 50 years or people in their teens (Canavan *et al.,* 2014). This may indicate that symptoms of IBS may remit as a person grows older. It is proposed that IBS is a disease of industrial development (Gwee, 2005; Grodzinsky *et al.,* 2012). In a community-based study, it was observed that in communities where lower number of people are involved in labor, the prevalence of IBS is high, because excessive work routine increases physical activity reducing chances of IBS(Gwee, 2005). Stress and anxiety that comes with the pressure of upscaling of living conditions may be a basic cause of the onset of IBS (Gwee, 2005).

2.3. Diagnosis of Irritable Bowel Syndrome

In 2016, a new criteria (Rome IV) was published for the diagnosis of gastroenterological disorders; this was an evolution of the old criteria Rome III (Malagelada, 2006; Drossman, 2016). Rome IV and Rome III have many differences such as Rome IV criteria established a link between functional gastrointestinal disorders and brain and defined GI tract disorders as problems associated with the gut-brain axis (Drossman, 2016). Many gastrointestinal disorders are diagnosed by Rome criteria such as irritable bowel Syndrome (IBS), reflux hypersensitivity syndrome (RHS), cannabinoid hyperemesis syndrome (CHS), opioid-induced constipation (OIC), and narcotic bowel syndrome (NBS), etc. (Drossman, 2016). The Rome IV for IBS is described below:

2.3.1. Rome IV Criteria

According to Rome IV standards, a patient would be diagnosed as having IBS if he/she experiences periodic abdominal pain or uncomfortable sensation in the abdomen once a week in the last three months accompanied by the following symptoms (Koppen *et al.,* 2017):

- Problems related to passage of stool.
- Problems associated with a change in the rate of passing the stool.

- Problem associated with a change in the physical appearance of the stool.

On the basis of bowel movement, IBS has been divided into four subtypes i.e. IBS-C (constipation), IBS-D (diarrhea), IBS-M (both constipation and diarrhea) and IBS-U (unidentified) (Drossman, 2016).

2.4. Red Flags in Irritable Bowel Syndrome

The subtypes of IBS (IBS-C. IBS-D, IBS-M, and IBS-U) are classified on the basis of the difference in symptoms (Drossman, 2016). However, with some expectation, there are some common symptoms shared among all of the subtypes. The shared symptoms are discussed below:

1. It is known that the brain and the gut are directly linked through the vagus nerve (Carabotti *et al.,* 2015). However, in IBS, this link gets disturbed leading to uncoordinated muscle movement in the large intestine causing pain. Pain usually disappears after a bowel movement (Fichna and Storr, 2012).
2. In IBS-D (Diarrhea) loose, watery and mucus-filled stool is very common, which leads to a sudden urge of passing stools.
3. As many as 50% of IBS patients are diagnosed with IBD-C (constipation). Constipation in IBS-C is accompanied by severe pain in the lower abdomen, which disappears after a bowel movement.
4. In IBD-M (Mixed), patients usually experience alternative cycles of severe diarrhea and constipation accompanied by pain in the lower abdomen.
5. Flatulence is another very common and nagging symptom of IBS. It is mainly experienced due to disturbed digestion in the intestine due to the modified gut microbiota.
6. Up to 70% of patients of IBS report food intolerances (Monsbakken *et al.,* 2006). These may not be categorized as food allergies because they don't start prompt systematic allergy cycles; instead, they just alter the bowel movement of the patients (Monsbakken *et al.,* 2006).
7. Patients with IBS usually experience lethargy and insomnia due to painful gastric problems.
8. In IBS patients, anxiety and stress are generally observed (Cole *et al.,* 2006). It has been proved that microbes in gut help in the modulation of mood by releasing some compounds which are the building blocks of serotonin (Cole *et al.,*

2006). In IBS, the disturbance of gut microbiota leads to a decrease in the level of serotonin in the body leading to stress, anxiety, and depression (Cole *et al.,* 2006).

2.5. Pathophysiology of IBS

IBS is a gastrointestinal disorder that has unclear pathology. Scientists around the world are working hard on establishing a general understanding of the pathophysiology of the IBS. Recently, it has been established that IBS may arise due to the dysregulation of important neurological points of the brain-gut axis, with alterations in many major points of the enteric, autonomic and/or central nervous systems, or altered neurological connections between these systems (Cole *et al.,* 2006). These modifications lead to disturbance in the local gut environment by modifying secretions, motility and food sensitivity leading to hallmarks of IBS such as gassiness and pain in the lower abdomen along with depression and anxiety (Malagelada, 2006). Patients with some IBC subtypes result in low-grade inflammation and defective immunological functions (El-Salhy *et al.,* 2013).

The IBS can be divided into two subsets: sporadic (nonspecific) and post-infectious (PI-IBS) (El-Salhy *et al.,* 2013). Sporadic IBS produces symptoms in patients without any previous underlying gastrointestinal infections (El-Salhy *et al.,* 2013) while PI-IBS, as the name indicates, starts after gastroenteritis (Thabane and Marshall, 2009).

The etiology of IBS is unknown, but its pathophysiology is a result of many contenders such as genetic factors, diet and nutrition, small and large colon bacterial flora, low-grade inflammation, and alterations in the function of the gastrointestinal endocrine cells (Lee and Park, 2014). In the paragraphs below, we discuss different aspects of the pathophysiology of IBS.

2.5.1. Pathophysiology: A Classical Approach

Over the past decade pathophysiology of IBS has focused on colonic dysmotility, visceral hypersensitivity, and the brain-gut interaction (Lee and Park, 2014). Alteration in the gastrointestinal movement is perceived as one of the essential pathological instruments in IBS, yet it does not completely correspond with symptomatic bowel problems. Colonic motor action of an IBS patient is different from healthy people. In the latter, high-amplitude propagated contractions (HAPCs) drive non-transmitted and periodic contractions and movement of intestinal contents

while in IBS patients, HAPCs occur repeatedly, leading to recurrent bowel moment that may cause watery stools in IBS-D patients. On the other hand, rare occurrence of HAPCs leads to slow bowel movement causing constipation in IBS-C patients (Lee and Park, 2014). However, there is incomplete scientific data to develop a link between colonic motility and IBS subtype. There may be some other diseases of the gut that show symptoms similar to that of non-diarrheal IBS so it is usually suggested that test for the proper function of gut i.e., anorectal function tests, should be considered.

Visceral hypersensitivity is also considered as a primary factor leading to the development of IBS (Lee and Park, 2014). It causes abdominal discomfort and intestinal motor disorder, which progresses towards the changes in the patterns of stool passage (either diarrhea or constipation) (Barbara *et al.,* 2004). It includes the sensitivity of rectal muscles, upper digestive tract, and colon (Posserud *et al.,* 2007). Rectal hypersensitivity and an increase in the sensitivity of the upper digestive tract after food intake is found to be common in IBS patients (Posserud *et al.,* 2007). Visceral sensitivity is also triggered due to stress (Posserud *et al.,* 2007). Generally, both extrinsic and intrinsic factors play a role in visceral hypersensitivity (Lee and Park, 2014).

Gut-brain axis is a major pathological entity in IBS. Research has revealed that lower colonic distention engages specific brain segment that generates pain (Mertz *et al.,* 2000). In IBS, it has been seen that this brain segment is more engaged as compared to in a normal person (Mertz *et al.,* 2000). Furthermore, there are certain hormones released under the control of the brain, which are usually disturbed in IBS patients (Mertz *et al.,* 2000).

2.5.2. Pathophysiology: Modern Approaches

In the previous segments, we have discussed old school approaches used to evaluate the pathophysiology of the IBS. However, with the advent of knowledge, a new concept has been introduced such as inflammation, post-infection low-grade inflammation, immunological and genetic factors, dietary habits, changed gut microbiota, and fault in enteroendocrine cells (Lee and Park, 2014). In the following paragraph, we would discuss these factors briefly.

Inflammation is the most common indicator of IBS (Cremon *et al.,* 2009). Colonic biopsies of IBS patients showed clear indications of inflammation such as triggered immune system cells, including T-lymphocytes, neutrophils, and mast cells in the mucosal layer of the intestine (Cremon *et al.,* 2008). Subsequently, some studies indicate high levels of mast cells in the epithelial layer of the gut of the IBS patients (O'sullivan *et al.,* 2000). Presence of mast cells in the gut mucosa is linked with a lot of functions e.g., healing of wounds, shielding against intruders, and increased in the sensitivity of GI tract mucosal layer (O'sullivan *et al.,* 2000). Mast cells are well-known for their release of inflammatory and immune mediators through degranulation, which recruits other immune cells which further enhance inflammation of the GI mucosal lining (da Silva *et al.,* 2014). Studies have shown that high level of mast cells in the gut correlates with certain symptoms of IBS i.e. abdominal pain or discomfort and flatulence (O'sullivan *et al.,* 2000).

Other than Mast cell, activated T-lymphocytes are also present in epithelial biopsy samples of IBS patients (Lee *et al.,* 2008). Compared to healthy person, mesenteric plexus of IBS patient is more infiltrated by lymphocytes (Lee *et al.,* 2008). Furthermore, the level of activated T-lymphocytes is also increased in the colon and blood of IBS patients (Lee *et al.,* 2008). Since T-cells are involved in the activation of B-cells and macrophages for the killing of infected cells in the body, an increase in colonic T-cells is indicative of infection (Deenick and Ma, 2011). Increased level of pro-inflammatory cytokines in blood mononuclear cells are also indicative of immune activation in IBS (Cremon *et al.,* 2008). Gwee (2010) observed that acute gastroenteritis increases the chances of IBS by 10% to 15%; most patients of gastroenteritis recover but in some cases it leads to the onset of IBS or post-infectious IBS (PI-IBS). The pathophysiology of PI-IBS involves low-grade inflammation characterized by increased infiltration of T-lymphocytes in the gut mucosal lining, increased mast cells and increased release of pro-inflammatory cytokines (Lee *et al.,* 2008; Gwee, 2010; Lee and Park, 2014).

Genetic pre-deposition also plays a significant part in IBS. Many studies have shown that polymorphism of certain genes is prevalent among IBS patients. Serotonin is a neurotransmitter, also known as serotonin, which is produced by the cells of the gut epithelial layer. It has many functions such as modulation of appetite and digestion, memory development, regulation of mood and social interactions, and

sleep-wake cycle (Lucki, 1998). Serotonin transporter (SERT) gene polymorphism (SLC6A4) has been shown to be associated with IBS (Kumar *et al.,* 2012). Initially, it was believed that it plays no part in the development of IBS and its polymorphisms depend upon environment and ethnicity but recent studies show otherwise saito.

Similarly, G-protein is known for its major role in signal transduction particularly by ligand-receptor binding (Saito *et al.,* 2007) coded by GNbeta3 genes. GNbeta3 polymorphism has also been associated with the development of IBS in some recent studies (Andresen *et al.,* 2006; Saito *et al.,* 2007). A neuropeptide S (NPS) is a 20 amino acid peptide which binds directly to neuropeptide S receptor (NPS1R), which in turn regulates the production of many important compounds such as somatostatin, peptide YY and vasoactive intestinal peptide (Camilleri *et al.,* 2014). Polymorphic variants of NSPR1 are found to be associated with the development of the IBS (Camilleri *et al.,* 2014). Endocannabinoid anandamide is a complex system involved in the modulation of many important tasks of the gut such as movement of the gut, gut sensation, gut secretion, and inflammation (Camilleri *et al.,* 2014). Endocannabinoid anandamide functions can be attenuated by fatty acid amide hydrolase (FAAH) (Camilleri *et al.,* 2014). In IBS-D, decreased transit time is associated with single nucleotide polymorphism (SNP) of FAAH (Camilleri *et al.,* 2014).

Enteroendocrine cells are present in the mucosa of the gut (El-Salhy *et al.,* 2010). These cells secret a number of important compounds that are involved in chemical sensation, modulation of gut motility, secretions, sensation of toxins and protection of gastrointestinal tract (El-Salhy *et al.,* 2017). They secrete important bioactive compounds i.e. gastrin, secretin, stomatostatin, cholecystokinin, chromogranins and serotonin (Kidd *et al.,* 2008; Lee and Park, 2014; El-Salhy *et al.,* 2017). The Most dominant enteroendocrine cell of the gastrointestinal tract is an enterochromaffin cell, which is scattered throughout the epithelial lining of the gastrointestinal tract (El-Salhy *et al.,* 2017). Enterochromaffin cells are specialized in the formation, storage, and secretion of serotonin in the gut lumen in response to external stimuli. It has been reported that secretion of serotonin increases in IBS-D and decreases in IBS-C (Kidd *et al.,* 2008). Other than serotonin, levels of chromogranin and secretogranin are also disturbed in IBS (Mazzawi *et al.,* 2015). Gut microbiota also contributes to the development of the IBS which is discussed in the coming section.

2.6. Normal Microbiota of Human Gut

The gut microbiota of humans keeps on varying with age and these variations have an effect on human health status. Until 1970s, culture-based methods were the only option for studying the microbiology of the gut. Recently, however, culture-independent techniques are available to gain actual knowledge on microbial communities in a specific environment. These molecular-based techniques have also confirmed the difference in the gut microbiota of infants, toddlers, adults and the elderly (Odamaki *et al.,* 2016). The mode of birth greatly affects the gut microbiota of the new born. Baby born through vaginal delivery would have dominating microbiota of the vaginal origin while baby delivered through caesarian section would have dominating microbiota of skin (Biasucci *et al.,* 2010). Subsequently, when child shifts from liquid to a solid diet, there is a significant change in the gut microbiota (Cabrera-Rubio *et al.,* 2012). The bacterial phyla evolve towards adult-like composition within 3-4 years after birth. However, some recent reports indicate that gut microbiota are not established even at the age of 5 years (Cabrera-Rubio *et al.,* 2012). After the stability of microbiota at the early stage of life, a drastic shift in the microbial composition has been observed at the later stage of the host (Odamaki *et al.,* 2016).

2.7. Gut Microbiota and Irritable Bowel Syndrome

A complex ecosystem of microbes containing approximately 1 x 10^{12} bacterial cells residing inside the human gut is called human gut microbiota (Faith *et al.,* 2013). A healthy person harbors approximately 195 different bacterial strains belonging to 101 species (Faith *et al.,* 2013); Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes are dominating phyla in a healthy gut (Lopez-de Los Santos *et al.,* 2012). The health of the gut is associated with large diversity and stability of the microbial community in the gut. With age, the diversity among microbial community decreases and it becomes unstable (Lozupone *et al.,* 2012). This microbial association in the gut is very important for the wellbeing of the host. It plays a major role in the gut such as release of energy from the digested food, making available nutrients for absorption, development of gut physiology, and protection from pathogens by competing with them for food and space (Lozupone *et al.,* 2012).

Butyrate is a very important short-chain fatty acid required for the proper growth and development of gut epithelium (Guilloteau *et al.,* 2010). Bacteria residing in the gut

are capable of modulating human nervous system by producing precursors such as tryptophan and neurotransmitters such as γ-aminobutyric acid (Williams *et al.,* 2014). Interaction of gut microbiota and the gut mucosal immune system is critical during early life, which continues throughout life (Lozupone *et al.,* 2012). This huge diversity of gut microbiota is very well tolerated by intestinal epithelial in the healthy state as they both have co-evolved together (Lozupone *et al.,* 2012). The stable presence of gut microbiota is important for normal physiological functions (Lozupone *et al.,* 2012). Stress, immune modulations and changes in the microbial composition lead to the disturbance of this balance between host and microbes leading to dysbiosis (Lozupone *et al.,* 2012).

2.7.1. Factors Modulating Gut Microbiota

Age

IBS is common in all ages, from children to older adults. However, age has no prominent difference in the onset and prevalence of different IBS subtypes (Odamaki, According to one study, 50% of IBS cases occur before age of 35. Then, the prevalence gradually decreases as the age increases. The prevalence is 25% after 50 years of age. These data suggest that IBS symptoms are reduced over time contradicting the belief that IBS is a chronic, life-long condition. The intensity of symptoms varies with age. Patients over 50 years of age report mild abdominal pain but the quality of life is worsened as they move to 65 years of age because symptoms of IBS may last longer than a year (Shah, Walker *et al*. 2019).

The progression of human gut Microbiota changes with age in terms of diversity and variation(Biagi, Candela, Fairweather-Tait, Franceschi, & Brigidi, 2012). Microbiota established at time of birth depends upon mode of delivery i.e., C-section or vaginal. According to recent studies, CFU for different body organs are: Mouth 105–108 CFU/ml, Stomach <104 CFU/ml, Duodenum 103–104 CFU/ml, Jejunum 103–105 CFU/ml, Ileum 107–108 CFU/ml and Colon 1011–1012 CFU/ml. Breast feed has higher numbers of *Bifidobacterium and Ruminococcus.* At early childhood, facultative anaerobes such as *Enterobacteriaceae,* streptococci and staphylococci are among the most prevalent groups(Marques *et al*., 2010).

Type of microorganisms present at time of birth depends also on hospital hygienic conditions during childbirth; because of better hygienic environment, exposure to

bacteria is reduced. In C-section birth, skin-derived staphylococci are the first colonizers of the infant gut and *Enterobacteriaceae,*and mother's intestinal and viginal microbiota are less dominant(Morelli, 2008). On the other hand, the vaginal born infants have dominant maternal vaginal and intestinal microbiota. Microbial diversity remains different in early weeks of life(Azad *et al*., 2013). Prominent factors which decide microbial diversity in early childhood include gestational age, use of antibiotics, and mode of feeding (breast vs bottle feeding); breast feeding has more Bifidobacterium and Ruminococcus(Yatsunenko *et al*., 2012a) but low numbers of *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis* and lactobacilli than bottle fed babies(Turroni *et al*., 2012).

Formula-fed babies have more complex bacterial diversity including enterobacterial genera, Streptococcus, Bacteroides, Clostridium, Bi(Morelli, 2008)fidobacterium and Atopobium(Penders *et al*., 2006). After introduction of solid foods, this gut microbial diversity changes further (Bezirtzoglou, Tsiotsias, & Welling, 2011). During adult hood the microbiota is usually stable unless there is a disturbance in external conditions (e.g., disease). At an old age, the stability of gut microbiota starts decreasing along with a reduction in variation. All of this depends on residence cohort, geographical location and detection methods used(Mueller *et al*., 2006). Researchers are now working on common core which will help them to see overall difference among age progression and decrease in microbial diversity(Biagi *et al*., 2010).Some of the studies show a decrease in diversity of Bifidobacteria and Bacteroides. The metabolic pathways efficiency also decreases in old age, which reduces amylolytic activity of microorganisms resulting in lower number of available short chain fatty acids(Mazzawi *et al*., 2019).

With age, the number of facultative anaerobes, fusobacteria, clostridia and eubacteria increases which leads to an increase in proteolytic activity. A recent study by ELDERMET consortium on comparison of gut microbiota of young and elderly subjects revealed the dominance of Bacteroidetes in the elderly while the microbiota of younger subjects was dominated by Firmicutes(Keebaugh, Williams, & William, 2019). In contrast, another research group concluded that there was no significant difference in variation of gut microbiota in young and old(Claesson *et al*., 2011). These results indicate that gut microbial diversity may depend upon country related variations as well as on diet (Tang *et al*., 2012).

Diet

Diet is an essential factor in modulation gut microbiota. It provides nutrition to both host and microbes for growth and development. Normal human diet contains a lot of such compounds which cannot be digested by the human body due to lack of enzymes needed for such digestions. Usually, microbes residing in the gut help in their digestion and release of energy helping maintain energy balance. It has been estimated that on average 40%-60% such indigestible compounds reach the human colon. These compounds include resistant starches, complex polysaccharides, and non-digestible carbohydrates, dietary proteins (e.g. collagen and elastin) as well as various secondary plant metabolites (Conlon & Bird, 2015). These compounds than digested through bacterial biotransformation and fermentation (Halmos, Power, Shepherd, Gibson, & Muir, 2014).

Bacterial genome is designed to metabolize carbohydrates efficiently i.e. efficiently switching between different carbohydrates source. Type of dietary components reaching the colon undigested defines the human gut microbiota. *Bacteroides* have the highest number of carbohydrate metabolizing enzymes so they are most abundant in the gut microbial community (El Kaoutari, Armougom, Gordon, Raoult, & Henrissat, 2013). Diet rich in probiotics increase relative abundance of *Bifidobacterium* in the gut. Resistant starches fermented by gut microbes into SCFAs which help in the development of gut mucosal layer and normal functioning of the gut (Sun, Wu, Liu, & Cong, 2017).

Lastly, it has been studied that divers dietary pattern contributes towards the diversity of microbiota in the gut while selected diet may target the increase in the relative abundance of the single microbial genera. Diverse microbial community contributes to strong gut immunity, increased diversity of metabolized compounds and improve the overall health of the host (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012b). Carbohydrate intake enhance production of SCFA by microbes due to which intestinal PH decreases ,which results in preventing growth of pathogenic bacteria especially E.coli and other enterobacteriaceace. (Kasubuchi, Hasegawa, Hiramatsu, Ichimura, & Kimura, 2015).

Diseases

Diseases such as inflammatory bowel disease (IBD) and inflammatory bowel syndrome (IBS) play a major role in the modulation of gut microbiota. In IBD such as ulcerative colitis (UC) and Crohn's disease (CD), mostly affects lower ileum and colon. In IBD it has been shown that the level of phylum Firmicutes decrease with the increase of phylum Bacteriodetes and facultative anaerobes (Enterobacteriaceae) in the gut. Even pathogenic *E.coli* strain has also been detected in the gut of IBD patients. Besides *E.coli*, an increased level of *Clostridium difficile* has been detected in the relapse of both forms of IBD. Level of *F. prausnitzii* decreases during IBD even it is prescribed as probiotic to cure the dysbiotic condition (Kasubuchi *et al*., 2015). In IBS, gut microbiota also changes which will be discussed in the next section.

2.8. Gut Dysbiosis in Irritable Bowel Syndrome

Studies showed that changes in microbial stability occur both in the small gut and large gut and contribute towards symptoms of IBS. For the sake of study model, gut microbiota is divided into two parts i.e. luminal microbiota and mucosal-associated microbiota. Luminal microbiota through its metabolic activity plays a significant character in the symptoms of IBS while mucosal-associated microbiota helps in immune modulation of the host (Kasubuchi *et al*., 2015). Studies suggest a shift in the relative abundance of the bacterial genus in large colon lumen when a person is developing IBS but up to date dysbiosis in IBS is still vague. Studies showed that the relative abundance of Firmicutes increases specially Ruminococcaceae spp. and Clostridium cluster XIVa while relative abundance of Bacteroides decreases (Claesson *et al*., 2012).

Relative abundances of *Lactobacilli, Coliforms*, and *Bifidobacteria* are low while relative abundance of *Pseudomonas* and *Enterobacteria* increases in IBS patients. Mucosal-associated microbiota involves clostridia cluster. IBS patients have an increased relative abundance of clostridia (Rajilić–Stojanović *et al*., 2011).Other than modifications of large colon microbiota, overgrowth of small gut microbiota is also a characteristic of IBS (Ghoshal, Shukla, & Ghoshal, 2017). Gram-positive and anaerobic bacteria harbors small intestine. There are many factors that prevent overgrowth of small intestine microbiota as small intestine microbiota overgrowth

leads to abdominal discomfort, bloating and flatulence. There are many factors that maintain the hemostasis of small gut microbiota. That may include bile production having anti-bacterial activity, peristalsis movement preventing attachment of bacteria to the epithelial wall, humoral and cellular defense, production of mucin and defensins that prevent the growth of pathogenic bacteria, and presence of ileocecal valves that prevent reflux of microbes from large gut to small gut.

Studies showed that the presence of small intestine bacterial overgrowth (SIBO) among IBS patients varies between 4% and 78% (Nucera *et al*., 2005). Such a large difference is due to the geographical region of study, a method of diagnosis and criteria of diagnosis. In SIBO, increased growth of gram-positive is due to decreased release of gastric juice and increased growth of coliform is due to increased retention time (Ghoshal et al., 2017). *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter lwoffii*, *Staphylococcus species*, *Klebsiella pneumonia*, *Streptococcus species*, *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Enterococcus faecium is detected in a jejunal aspirate of SIBO samples.* Gram-negative bacilli and Enterobacter are detected in the jejunal aspirate of IBS patients (Ghoshal, 2020).

2.9. Short Chain Fatty Acids (SCFA) and Gut Microbiota

SCFAs are the end products of bacterial fermentation of non-digestible carbohydrates that escape digestion absorption in the small and large colon. The major SCFAs are acetate, butyrate, propionate, formate, and lactate. Lactate mostly further converted into acetate and butyrate through cross-feeding mechanism while formate is involved in the methanogenesis. There are also branched SCFAs that are produced through fermentation of amino acids in the large gut. Production of acetate is distributed among large bacterial community while that of propionate and butyrate is somewhat substrate and species-specific (Raman, Ambalam, & Doble, 2016). *Akkermansia municiphilla* is recognized as propionate producer due to the presence of a metabolic pathway that converts deoxy sugars (fucose and rhamnose) into propionate through 1, 2-propanediol (Louis & Flint, 2017). *Ruminococcus bromii* is responsible for the fermentation of resistant starches eventually producing butyrate in the gut. Similarly, *Faecalibacterium prausnitzii, Eubacterium rectale,* and *Eubacterium hallii* are also

involved in the production of butyrate (Martínez, Kim, Duffy, Schlegel, & Walter, 2010).

2.10. Beneficial Effects of SCFAs

SCFAs has many beneficial effects on the human physiology which are discussed below:

2.10.1. Gut Integrity

SCFAs plays a major role in maintaining gut integrity. Especially butyrate is used as fuel by gut colonocytes to divide and maintaining healthy gut epithelium (Nofrarías, Martínez-Puig, Pujols, Majó, & Pérez, 2007). Paradoxically, butyrate induces proliferation of colonocytes to maintain gut health in the normal subject while on another hand in colorectal cancer it induces apoptosis and cell death (Alex *et al*., 2013). As we know that gut epithelial cells are strongly bound to each other due to the presence of tight junction proteins (TJP) between cells making epithelial impermeable to luminal contentd Increased permeability cause transfer of bacteria or bacterial membrane components (lipopolysaccharides LPS) which binds to Toll-like receptors 4 (TLR4) on the cells and initiate inflammatory cascade in immune cell. It further initiates downstream inflammatory cascade which involves transcription regulators such as nuclear factor kappa b (NF-kB) and mitogen-activated protein kinase (MAPK) which increase production TNF-alpha and IL-6. Butyrate increases expression of TJP regulatory proteins i.e. claudin-1 and Zonula Occludens-1 (ZO-1) which increase the integrity of gut epithelia(den Besten, van Eunen, *et al*., 2013).

2.10.2. Lipid Metabolism

Three major SCFAs i.e. acetate, propionate and butyrate are produced in the colon. Butyrate immediately absorbed by colonocytes while acetate and propionate enter portal circulation. Acetate and propionate both play a major role in lipid metabolism through Ffar2 and Ffar3 receptors and AMP-activated protein kinase (AMPK)(Puertollano, Kolida, & Yaqoob, 2014). Binding of SCFAs to receptor increase the activity of AMPK which induce expression of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 alpha which further regulates the transcriptional activity of peroxisome proliferator-activated receptor (PPAR) alpha, PPAR-beta, PPAR-gamma, liver X receptor (LXR), and farnesoid X receptor (FXR) all of which play important role in glucose, lipid, and cholesterol metabolism(Jäger,

Handschin, Pierre, & Spiegelman, 2007). SCFAs induce AMPK activity either directly by AMP/ATP ratio or indirectly through Ffar2 dependent leptin pathway. Many studies approved indirect pathway where leptin is a hormone that increases AMP/ATP ration and induces fatty acid oxidation in liver and muscles (Fig 4)(den Besten, van Eunen, *et al*., 2013).

In brown adipose tissues, SCFAs increase expression of PGC-1 alpha and uncoupling protein (UCP)-1 which in turn increase fatty acid oxidation (van Eunen *et al*., 2013). While in white adipose tissues, SCFAs start many pathways: 1) increase Ffar2 dependent leptin which releases into plasma and increases AMP/ATP ration in muscles and liver leading to increased fatty acid oxidation 2) increase ATP/cAMP ratio inhibiting protein kinase A which in turn inhibit hormone-sensitive lipase (HSL) which reduce lipolysis and reduce plasma free fatty acids 3) decreasing insulin sensitivity and reducing fat accumulation. All these metabolic networks lead to decreased lipolysis leads to decreased free fatty acids and ultimately low body weight (den Besten, Lange, *et al*., 2013).

2.10.3. Glucose Homeostasis

The large intestine has recently been identified as a gluconeogenic organ and it has been observed that intestinal gluconeogenesis (IGN) has a role in energy regulation and glucose homeostasis. Butyrate and propionate both play a major role in IGN with butyrate induce expression of the certain gene in IGN while propionate act as a substrate. Additionally, propionate has seen in the induction of peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) hormones which are considered as hunger suppressing hormones and plays a major role in glucose homeostasis (Fig 5) (Zhou, Martin *et al*. 2008, Tolhurst, Heffron *et al*. 2012).

PYY produced by colonocytes released in plasma and trigger glucose uptake in muscles while GLP-1 produced by colonocytes released in plasma and upregulates insulin expression while downregulates glucagon expression (Zhou, Martin *et al*. 2008). While SCFAs downregulates gluconeogenesis in the liver by up regulating the AMP/ATP ratio, so SCFAs are very important in glucose homeostasis any change in gut microbiota leads to reduce production of SCFAs, which in term effect body normal functions.(den Besten, van Eunen *et al*. 2013).

2.11. Short-Chain Fatty Acids and Irritable Bowel Syndrome

In the previous segment, the relationship between gut microbiota and IBS has been discussed. It was examined that dysbiosis in the gut leads to symptoms associated with IBS. And it is also discussed in the previous segment that gut microbiota is involved in the fermentation of non-digestible carbohydrates in the gut and produces SCFAs as by-products. Acetate, propionate, and butyrate are three major SCFAs that play a major role in the normal gut function. Studies have been carried out to calculate the level of SCFAs in IBS patients (Farup, Rudi *et al*. 2016). It has been observed that the level of acetate and propionate is higher in the IBS patients while that of butyrate is towards the lower side in the IBS patients (Farup, Rudi *et al*. 2016). In a study, a higher level of propionate and lower level of butyrate was calculated in the IBS patients than the normal person. They calculate propionate/butyrate ratio and propionate-butyrate to differentiate between normal subject and IBS patient (Farup, Rudi *et al*. 2016). They found that Pro-But is the best criteria to diagnose IBS (Farup, Rudi *et al*. 2016). If the Pro-But value is <-0.13 IBS is excluded while if it is >0.46 IBS is suspected (Farup, Rudi *et al*. 2016). This study purposed that levels of SCFAs in the feces can be used as a biomarker for the diagnosis of IBS and it is a noninvasive and reliable method for the diagnosis.

2.12. Butyric Acid in the GUT

Butyric acid is a byproduct of microbial fermentation in large colon, or non-digestible carbohydrates, resistant starches, oligosaccharides, sorbitol, mannitol, lactose, and non-starch polysaccharides (McOrist, Bird *et al*. 2011). Different grains and seed, fruits, and vegetables are rich in resistant starched and considered as butyrogenic in nature (McOrist, Bird *et al*. 2011). The human gut is found in the house of many butyrate-producing bacterial species. Bacterial species related to Clostridial cluster VI. *Eubacterium* spp and *Fusobacterium* spp constitute 5-10% of the total species that involve in butyrate production (Louis and Flint 2009, McNabney and Henagan 2017). Besides, *Bifidobacterium spp* and *Faecalibacterium prausnitzi* produce butyrate by a cross-feeding mechanism (Moens, Weckx *et al*. 2016, McNabney and Henagan 2017). While, *Butyrivibrio* spp., *Megasphaera elsdenii, Mitsuokella multiacida, Roseburia intestinalis*, and *Eubacterium hallii* are other bacterial species present in the gut that are involved in butyrate production

(McNabney and Henagan 2017). Butyrate produced in gut impart many beneficial effects on the human body. Positive effects of butyrate are discussed in the next segment.

2.13. Valuable role of Butyrate in Human Body

Butyrate is a holy grail produced by human gut microbiota. It has amazing effects on the human body which are discussed below: Butyrate act as a chemotherapeutic agent in colon cancer (Bates 2012). The mechanism behind this lies in the cancer cells energy obtaining system. Cancer cells obtain energy through anaerobic glycolysis even in the presence of high level of O2. In the presence of anaerobic glycolysis, fatty acid oxidation also decreases due to which butyrate accumulate in the cytoplasm. Cancerous colonocytes don't use butyrate as an energy source.

High level of butyrate in cytoplasm act as histone de-acetylation inhibitor causing inhibition of transcription in cancer cells ultimately inducing apoptosis in cancer colonocytes (Vander Heiden, Cantley *et al*. 2009). Besides the above-mentioned mechanism, butyrate also induces apoptosis by binding to a type of G-protein coupled receptor GPR109A (Thangaraju, Cresci *et al*. 2009). Nicotinate is the ligand of the GPR109A receptor while butyrate also activates with less affinity but colon lumen has enough butyrate which is able to activate to maximum (Elangovan, Pathania *et al*. 2014). In most of the cancers including colon cancer its expression is silenced (Elangovan, Pathania *et al*. 2014). But by re-expressing it in the cancer cells in the presence of butyrate induces apoptosis in the cancer cells (Thangaraju, Cresci *et al*. 2009).

Another beneficial property of butyrate is its ability to mediate the immune response in the gut epithelial layer. Chang *et al*. have thoroughly studied the effect of butyrate on macrophages residing in lamina propria in the gut (Chang, Hao *et al*. 2014, McNabney and Henagan 2017). Out of all three SCFAs (acetate, propionate, and butyrate), the only butyrate has shown to decrease the level of pro-inflammatory cytokines IL-6 and IL-12p40 in addition with antimicrobial nitric oxide (NO) (Chang, Hao *et al*. 2014, McNabney and Henagan 2017). Butyrate specifically affects the macrophages and secondly, it has no effect on the downstream signaling pathway of lipopolysaccharide (LPS) induce Toll-like receptor 4 (TLR-4) (Chang, Hao *et al*. 2014, McNabney and Henagan 2017). In this way, butyrate mediates the immune

response thus protecting beneficial microbiota while immune system only targets harmful microbes in the gut.

2.14. Butyrate and Irritable Bowel Syndrome

Studies have shown that butyric acid has a positive role in alleviating symptoms of IBS(Załęski, Banaszkiewicz *et al*. 2013). Butyric acid in its pure form is not eatable due to its pungent smell and it also absorbed in the upper digestive tract rendering its useful effects in the lower gastrointestinal tract (Tarnowski, Borycka-Kiciak *et al*. 2011). In a study, the different chemical combination of butyric acid has been used. One such combination is sodium butyrate which has shown very promising results (Tarnowski, Borycka-Kiciak *et al*. 2011). Administration of sodium butyrate capsule to IBS patients has shown a reduction in impulsive abdominal pain, post-prandial abdominal pain, and requirement after defecation during the 12^{th} week of treatment (Banasiewicz, Kaczmarek *et al*. 2012). In another study, triglyceride encapsulated butyric acid has been used for the treatment of IBS symptoms (Tarnowski, Borycka-Kiciak *et al*. 2011). This experiment ensured a slow release of butyric acid to the target site. Scarpellini *et al*. have deeply studied the effects of butyrate in the treatment of diarrhea-predominant IBS. In their study, they concluded that double coated tablets of butyric acid help in uplifting the symptoms such as flatulence, camp and abdominal pain in IBS-D patients (Scarpellini, Lauritano *et al*. 2007).

2.15. Techniques used to study gut microbiome

Previously most of studies used terminal restriction fragment length polymorphisms to assess the microbiome, as research continuous use of 16S ribosomal RNA (rRNA) gene become more popular beside these quantitative polymerase chain reaction, fluorescent in-situ hybridization, microarrays, denaturing gel gradient electrophoresis are some of common techniques used to study gut microbiome(Fraher, O'toole, & Quigley, 2012).

Chapter 3: Irritable Bowel Syndrome pathophysiology in selected patients from Rawalpindi/Pakistan

This study was conducted in Labortary for Microbial Food Safety & Nutrition, Faculty of Biological sciences, Department of Microbiology, Quaid-I-azam university, Islamabad, AFIP (Armed forces institute of pathology) combined military hospital (CMH) and gastroenterology department Military hospital (MH) Rawalpindi.

3.1. INTRODUCTION

Irritable bowel syndrome is a reverting functional bowel disorder, diagnose based on symptoms. Although symptoms are not specific in IBS and every individual may face different conditions, proposed diagnostic criteria is based upon symptoms occurrence and time duration (Hazaa and Lami 2018). Feeling of discomfort, cramping with defecation problems are some common features. (Cremonini and Talley 2005). Lack of any specific biomarker that can be used for the diagnosis of IBS, greatly affects the study of prevalence of IBS (Thompson, Heaton *et al*. 2000).

Prevalence studies in a community greatly depends upon the general mindset of the population related to seeking medical help and tools used for data collection (questioners, telephone calls, and mails etc.). Meta-analysis showed that Southeast Asia has the lowest prevalence of 7% while South America has highest of 21% (Canavan, West *et al*. 2014). Worldwide prevalence of IBS is calculated to be 10-15% while in Asia it is around 4.4 to 10% (Canavan, West *et al*. 2014). In Europe and North America 10-20% of population is affected with IBS. Global prevalence of IBS has variations depending upon criteria used for diagnosis like Manning, Rome I, Rome II, Rome III or Rome IV (Lovell and Ford 2012). One of the study conducted by (Sperber, Dumitrascu *et al*. 2017), analysis a large data of 41 countries and show prevalence of 1.1% in France and Iran to 35.5% in Mexico, there study also proved that diagnostic criteria effect prevalence rate like using Rome I prevalence was 6.7%;while Rome II showed 7.8%; Rome III showed 9.1%; Manning showed 8.3%; and no specified diagnostic criteria 12.8%.

Ratio of IBS prevalence was higher in female then in male (10.2% vs 8.8%). Worldwide South Africa has low prevalence of IBS 10-25%, while South America has highest prevalence 21% (Canavan, West *et al*. 2014). In Pakistani population prevalence of IBS was found to be 28.3%, with a predominance of 87 (85.29%) females (85.29%) over males (14.71%) (Naeem, Siddiqui *et al*. 2012),in another study on Pakistani population 2007 presented that 45% of 1084 participants in Bahawalpur and Karachi were diagnosed by IBS (Jafri, Yakoob *et al*. 2007).

According to recent criteria for IBS diagnosis patients are divided into three categories depending upon type of symptoms and stool form they experience. **IBS-C** patients have constipation like symptoms, common symptoms include abdominal pain and discomfort along with some changes in bowel function and may experience bloating and gas. Bowel functions changing include sometimes infrequent stool, hard or lumpy stools or sometimes it's more often psychologically having feeling of heaviness in bowel, symptoms are ongoing having chronic effect but sometimes it may come and go (Atluri, Chandar *et al*. 2014). **IBS-D:** Diarrhea predominant IBS is more prevalent, having diarrheal like symptoms associated with accelerated small bowel or colonic transit. Common symptoms include cramping, urgency, diarrhea and high colonic contractions (Camilleri, Atanasova *et al*. 2002).**IBS-M** is with alternating episodes of both diarrhea and constipation very quickly, studies have found that symptoms of abdominal pain and discomfort are more severe in IBS-M than any other IBS type (Drossman, Patrick *et al*. 2000).

3.1.2 : Factors effecting IBS

There are many key-players that contribute to the development of IBS such as age, genetic factors, gender and socio-economic condition of the patients. (Chang, Lu *et al*. 2010). Diet is an essential factor in modulation gut microbiota. It provides nutrition to both host and microbes for growth and development. Normal human diet contains a lot of such compounds which cannot be digested by the human body due to lack of enzymes needed for such digestions. Usually, microbes residing in the gut help in their digestion and release energy, helping them to maintain energy balance. It has been estimated that on average 40%-60% such indigestible compounds reach the human colon (Conlon and Bird 2015). These compounds include resistant starches, complex polysaccharides, and non-digestible carbohydrates, dietary proteins (e.g. collagen and elastin) as well as

various secondary plant metabolites. These compounds than digested through bacterial biotransformation and fermentation (Power, O'Toole *et al*. 2014). Diet rich in probiotics increase relative abundance of *Bifidobacterium* in the gut (Singh, Chang *et al*. 2017). It has been studied that divers dietary pattern contributes towards the diversity of microbiota in the gut while selected diet may target the increase in the relative abundance of the single microbial genera. Diverse microbial community contributes to strong gut immunity, increased diversity of metabolized compounds and improve the overall health of the host (Lozupone, Stombaugh *et al*. 2012).

Alteration in gut microbiota is an important factor in pathogenesis and pathophysiology of IBS, with age gut microbial diversity also changes, the transition of gut microbiota from infancy to the elderly is associated with the dominance of specific bacterial genera, common are *Bacteroides*, *Eubacterium, Megamonas*, *Peptoniphilus*, and *Clostridiaceae* related to elderly people; *Enterobacteriaceae* are related to infants and elderly; *Bifidobacterium* are related to infants or child; *Lachnospiraceae* are related to adult-associated and *Dorea* is unrelated to age. IBS is usually more common among 30-40 years age group, but studies also proved age effect on IBS onset (Odamaki, Kato *et al.* 2016).

Some of previous research papers found out relationship between physical activity and IBS symptoms, increase physical activity helps in reducing symptoms of IBS symptoms. Good physical activity also effect quality of life (Johannesson, Simrén *et al*. 2011).Physical activity decreases stress level so having a favorable effect on brain cells.(Dishman, Berthoud *et al*. 2006), but physical activity effect body after a long period and improvement in fatigue, depression and anxiety and quality of life will be observed, so consistence physical activity lower depressive symptoms and protect against IBS symptoms deterioration(Herring, Puetz *et al*. 2012).

Internationally IBS is more prevalent in women, according to one survey report its 1.5-3 folds higher, but when prevalence was observed region wise no significant sex difference was observed in most of regions like South Asia, South America, and Africa (Lovell and Ford 2012). One of the study carried out in Asia show no gender difference(Gwee, Lu *et al*. 2009), other regions having some sex related difference can be summarized as: India (female vs male): 7.9% vs 6.9%, 4.3% vs 4%, 3.2% vs 4.8%(Ghoshal, Abraham *et al*. 2013); Korea: 7.1% vs 6.0%; Hong Kong: 6.6% vs 6.5%; Pakistan: 13.1% vs 13.4%;

Taiwan: 21.8% vs 22.8%; Singapore: 7.8% vs 9.4%; Malaysia: 10.6% vs 10.5%; Japan: 6.5% vs 5.5%).28,36-38, while western population usually reports women have more IBS prevalence then male (Gwee, Gonlachanvit *et al*. 2019).

3.1.3 : Liver enzymes

There is a close relationship between gut microbiota and metabolic pathways, gut microbiota has a key role in progression of metabolic disorders like insulin resistance, studies now proved the role of gut microbiota in functional gastrointestinal disorders(FGIDs), (D'Aversa, Tortora *et al*. 2013). One of the important FGIDs which got affected by changing in gut microbiota is IBS; common symptoms includes pain in abdomin along with discomfort which leads to alteration of bowel habits (Agrawal and Whorwell 2006). Due to un clear etiology researches have given many hypothesis like gut microbial changes, dysfunctioning of brain-gut axis, and also changes in hormones of gut leads to IBS. Among these small intestinal bacterial overgrowth also plays role.chnages in production of liver enzymes due to overgrowth of bacterial strains also have role in IBS progression. (SIBO) (Parkes, Brostoff *et al*. 2008),(Singh and Toskes 2004). Changing in normal gut microbiota disturbs gut permeability which in terms can leads to non-alcoholic fatty liver disease (NAFLD) and metabolic derangement i.e., the gut-liver axis. In animals models NAFLD is induced by breakdown of intestinal barrier as a result of microbial product translocation (Szabo, Bala *et al*. 2010).The liver enzymes most commonly found are: Alanine transaminase (ALT),Aspartate transaminase (AST),Alkaline phosphatase (ALP), Gamma-glutamyl transpeptidase (GGT),sometime due to inflammation liver enzymes might go high.

Table 3.1: Liver enzymes range in normal human

Expected values	ALT U/L	ALP U/L	AST U/L
Male	7 – 40	90 – 360	< 34
Female	7 – 40	70 – 290	< 34

3.1.4. Complete Blood Count

This blood test is used for analysis of patient overall health and to detect wide range of disorders mainly related to blood e.g. anemia, infections or leukemia.in IBS patients CBC is used to eliminate chances of any other infections normally related to digestive systems. (Guilera, Balboa *et al*. 2005), blood profile of IBS patient should be normal in order to eliminate presence of any other infection. (Riedl, Schmidtmann *et al*.

2008).CBC includes counting of number of, red blood cells (RBC), white blood cells (WBC), hemoglobin in the blood, platelet count – these cells are responsible for clotting(Lovell and Ford 2012). Normal values for the CBC may vary slightly among different labs but are generally as follows:

Table 3.2: Normal values of CBC (Ford, Marwaha *et al.* 2010)

Test name	Normal Range	
Gender	**Male**	**Female**
RBC	4.7 to 6.1 million cells/mcL	4.2 to 5.4 million cells/mcL
WBC count	4,500 to 10,000 cells/mcL	4,500 to 10,000 cells/mcL
Hemoglobin	13.8 to 17.2 gm/Dl	12.1to 15.1 gm/dL

3.1.5. Vitamin D

Some scientist believe that food allergies and vitamin D deficiency also play a role in IBS severity (Fond, Loundou *et al*. 2014) .Some recent studies confirm the relationship between vitamin D level and IBS, as vitamin D has a very important role as an anti-inflammatory, and anti-microbial agent, due to these important role performed by vitamin D in body it is thought to have link with IBS. Vitamin D low level in body may initiates or aggravates IBS symptoms, (Agarwal and Spiegel, 2011; Dupont 2014).

Supplements with vitamin D improve symptoms of IBS as well as quality of IBS patients. However, similar studies are lacking in Pakistani population this study was conducted to check vitamin D level of IBS Pakistani population.(Sainsbury, Sanders *et al*. 2013).

3.1.6. Lipid profiling

The leading cause of death in world is dyslipidemia, as this increases amount of lipid in blood, (Adhyaru and Jacobson 2016). Many researches proved alterations in lipid profiles of gastrointestinal diseases patients e.g. in Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), lipid profiles of patients changes due to interactions of inflammatory cytokines, malnutrition, and malabsorption ,it can be due to intestinal damage, which results in decrease in total cholesterol and low-

density lipoprotein (LDL) levels and an increase in triglyceride and HDL levels compared to healthy controls (Levy, Rizwan *et al*. 2000).

No such studies are carried out in IBS patients but in patients with IBD total cholesterol and LDL cholesterol decrease with disease activity and they increase with IBD treatment with biologics. For total cholesterol: lipid profiling includes measuring, total cholesterol level, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (Biyyani, Putka *et al*. 2010).

Table 3.3: Lipid profile of normal human

	Lipid profiling		
Test	**Normal(mmol/L)**	**Borderline(mmol/L)**	**High(mmol/L)**
Cholesterol	3.6-5.2	10-11.1	>11.1
LDL	2.6-3.4	3.5-4.1	4.2 to 5
HDL	2.2	3.3	3.5
Triglycerides	1.7	1.7-2.1	2.2-5
VLDL	0.1-1.7	-	>2.1.7-2.11

3.1.7. SCAFS production by gut microbiota

Microbial communities residing in the gut produce many useful products such as short-chain fatty acids (SCFAs) as end-products of bacterial fermentation of complex carbohydrates in the large colon, which remains undigested in the small intestine. (Cantu-Jungles, Cipriani *et al*. 2017).

SCFA are characterized by their short carbon chain i.e. from C2 to C8. Acetate, propionate, and butyrate are the abundant SCFAs in the human gut up to 90 to 95 % of total SCFAs produced. SCFAs involved in a number of functions such as maintaining gut integrity, glucose metabolism, lipid metabolism, cholesterol metabolism, and appetite regulation.(Ríos-Covián, Ruas-Madiedo *et al*. 2016).

3.1.8. Aim and Objectives

The aim of the present study was to analyze role of atmospheric factors, blood parameters in IBS progression and to further optimize and validate an existing fast GC-MS based method to determine the concentration of SCFA in serum from human IBS subjects, the specific objectives of study are:

- To evaluate the IBS patients clinical epidemiological profile
- To determine hematology parameters of IBS patients

3.2. Materials and Methods

3.2.1. Selection of Patients

This is a cross-sectional study during the year 2017/2018. A multistage stratified random sample method was used. A total of 60 patients were selected. A standardized self-administered questionnare was used. It was administered under supervision of gastroenterologist. For the selection of patients, gastro OPD of local hospital was visited, patients were selected on the inclusion and exclusion criteria developed before handily according to the literature. Inclusion and exclusion criteria are discussed below, Inclusion criteria include the following considerations:

- All patients must fulfill Rome VI criteria i.e. periodic abdominal pain experienced once a week in the last three to four months. Problems experienced related to the frequency of stool passed and change in the appearance of stool.
- Complete blood report of the patient was examined. Anti-TTG, a test for celiac disease, should be negative to rule out chance of celiac disease. Complete blood count (CBC) should be normal. The patient should not have any underlying infection.
- Lactose and gluten intolerance test should be negative.
- No history of inflammatory bowel diseases (IBD) i.e. ulcerative colitis and Crohn's disease.

Exclusion criteria include the following considerations:

- Patient not fulfilling Rome VI criteria.
- Patient having a positive test for anti-TTG.
- Patient having lactose or gluten intolerance.

- Patient having a history of IBD.

3.2.2. Epidemiological data collection

Self-administered Questionnaire was developed to collect epidemiological data from patients, following factors are recorded using questionnaire:

Age, gender, medical history, duration of IBS, Dietary patterns of patient from last one year, Stress level and Physical activity. All these data were recorded and converted into numerical data by help of scoring sheath.

3.2.2. Sample collection

In this study, two types of samples were selected i.e. stool and serum sample. Stool samples were collected in a sterilized stool container from IBS patients. Whereas blood samples were collected in the serum-separating tube (SST) yellow cap tubes. Blood samples were immediately centrifuged to separate serum. Serum and stool were then stored at -20 till further process.

3.2.4. Scoring sheath

In order to analyze epidemiological data, we developed a scoring sheath, which make it easy to see correlation between different factors. Scoring sheath is conversion of non-numerical epidemiological data into numerical data, which includes different parameters i.e. time from which patient is having IBS, presence of any other gastrointestinal infection, IBS, or patient mood swings or anxiety due to IBS.

We have collected data of all patients using self-administered questionnaire. Four factors are considered as variable for developing scoring scale.

- ✓ Disease duration
- ✓ Presence of any other GIT infection
- ✓ IBS subtype
- ✓ Anxiety related to IBS

Table 3.4: Severity score calculation formula for selected IBS patients

S.Nu	Criteria	Severity Scoring scale				
1	**Disease duration**	**>1 year**	**1-5 years**	**5-10years**	**10-15 years**	**<15**
	Score	1	2	3	4	5
2	**Other GIT infection**	**Yes**	**No**			
	Score	1	0			
3	**IBS subtype**	**constipation**	**Diarrhea**	**Mixed**		
	Score	1	1	2		
4	**Depression**	**Low**	**Mild**	**High**		
	Score	1	2	3		

On basis of severity score calculation formula, patient's severity score is calculated.

Table 3.5: IBS patients showing different severity score, develop on basis of severity score

Patient ID	Severity score	Patient ID	Severity score	Patient ID	Severity score
IBS 1	4	**IBS 22**	8	**IBS 42**	6
IBS 2	8	**IBS 23**	7	**IBS 43**	7
IBS 3	6	**IBS 24**	3	**IBS 44**	5
IBS 4	5	**IBS 25**	5	**IBS 45**	4
IBS 5	8	**IBS 26**	6	**IBS 46**	4
IBS 6	6	**IBS 27**	6	**IBS 47**	5
IBS 7	6	**IBS 28**	7	**IBS 48**	5
IBS 8	5	**IBS 29**	8	**IBS 49**	10
IBS 9	9	**IBS 30**	7	**IBS 50**	9
IBS 10	5	**IBS 31**	6	**IBS 51**	7
IBS 11	6	**IBS 32**	7	**IBS 52**	4
IBS 12	5	**IBS 33**	6	**IBS 53**	6
IBS 13	5	**IBS 34**	5	**IBS 54**	6
IBS 14	7	**IBS 35**	7	**IBS 55**	5
IBS 15	5	**IBS 36**	9	**IBS 56**	5
IBS 16	9	**IBS 37**	5	**IBS 57**	9
IBS 17	6	**IBS 38**	6	**IBS 58**	8
IBS 18	5	**IBS 39**	4	**IBS 59**	5
IBS 19	6	**IBS 40**	9	**IBS 60**	6
IBS 20	6	**IBS 41**	7		
IBS 21	4				

3.2.4. Short Chain Fatty Acid Extraction from Serum

3.2.4.1. Chemicals and sample preparation

Three chemicals were used in the extraction of short chain fatty acids from serum. These were acrylic acid, metaphosphoric acid, and propyl format. Samples were preparedaccording to a protocol designed through literature (Skoglund 2016). According to the protocol, 200 µl of serum, 100 µl of 150 µM acrylic acid, and 100 µl of 1500 µM m- phosphoric acid added in microliter tube followed by vortex on high speed for 5 minutes.After that samples were centrifuged at 14000 rpms. The supernatant is separated in new microliter tube and 100 µl of pure propyl format is added followed by the second cycle of centrifugation at 1400 rpms. The supernatant is collected and directly analyzed by GC-FID.

3.2.4.2. Standard Curve formation and recovery

For the formation of standard curves, the standard for butyric acid was purchased. Six dilutions were prepared by dissolving standard into methanol. Six dilutions were 5 ug into 100 µl, 10 µg into 100 µl, 15 µg into 100 µl, 20 µg into 100 µl, 25 µg into 100 µl and 30 µg into 100 µl. A calibration curve was generated through GC-FID.

In the preparation of samples, propyl format is used which causes loss of SCFAs from the sample. That's why, the calculated amount of 10µg/ml of acrylic acid (Ac) was added into the sample before the propyl format according to the protocol (Skoglund 2016). After the estimation of SCFAs, the amount of acrylic acid was added in the amount of SCFAs detected by GC-FID.

Actual Concentration = Observed concentration + Amount of Ac added

3.2.5.2. Analysis of Serum Samples for Short Chain Fatty Acids

To qualitatively analyze the SCFAs in the serum GC-MS was used. In GC-MS, the initial oven temperature was 55ºC for 4 min, after that at the rate of 50ºC/min it is raised to 130ºC and maintained for 3.7 minutes. Ultimately, ramped up to 230ºC at the rate of 30ºC/min. 70 eV at the temperature of 250ºC was used for electron ionization (Skoglund 2016).

To determine SCFAs (quantity) in the sample's gas chromatography-flame ionization detector was used. The protocol used was reported in the literature (Baltierra-Trejo,

Sánchez-Yáñez *et al*. 2015). 1 µl of the sample was injected in the inlet valve fitted with the capillary column. Nitrogen (N_2) gas was used as carrier gas at a constant flow rate of 15.0 ml min^{-1}. An oven temperature of GC was maintained at 200ºC while that of FID was maintained at 250ºC. For the injector, the temperature started from 120ºC with a ramp of 10ºC until reached 200ºC was used (Baltierra-Trejo, Sánchez-Yáñez *et al*. 2015).For co-relation analysis, Pearson co-relation or product moment correlation coefficient (PMCC) by XLSTAT software was used.

3.2.5. Measurement of liver enzymes

3.2.6.1. Aspartate transaminase (AST)

Liver enzymes Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), these are measured using kit provided by ADIVA chemistry, kit is formulated for invitro determination aspartate transaminase AST, quantitative measurement of AST helps in diagnosis or monitoring of any abnormal liver functionaing. Reaction works on following principle:

340/410 nm absorbance wavelength is used to measure concentration of NADH, formed as a result. addition of ⌐ -ketoglutarate, the rate of absorbance decreases in proportional to AST activity. This can be shown as reaction form:

L-Aspartate + □-Ketoglutarate ----------AST--------- → Oxalacetate + L-Glutamate

Oxalacetate + NADH --------malate dehydrogenase >Malate + NAD

3.2.6.2. Alanine transaminase (ALT)

Alanine aminotransferase is measured using ADIVA chemistry kit for ALT, this is used for in vitro diagnosis and to study postnecrotic liver disorder, basic principle is:

Measurement of NADH by adddition of reagent α-ketoglutarate, absorbance decrease is proportional to ALT activity. Reaction equation is:

ALT + P5P → Activated ALT

L-Alanine + α-Ketoglutarate → Pyruvate + L-Glutamate

Pyruvate + NADH → Lactate + NAD

3.2.6.3. Alkaline phosphatase (ALP)

ADIVA chemistry kit is used for quantitative measurement of alkaline phosphate.in this method a substrate p-nitrophenyl phosphate is used, sample is added on substrate.DAE is then added which maintains reaction PH at 9.7-9.8,further magnesium ions are added to the buffer which helps in activation and stabilization of enzymes.during this reaction p-

nitrophenol is formed as alkaline phosphate hydrlyzes Pnpp,this yellow colour p-nitrophenol can be measured photometrically at 410/478 nm.

PNPP + D -⟶ P-Nitrophenol + P-DEA

3.2.7. Lipid profiling of IBS subjects

Siemens Medical Solutions Diagnostics chemistry analyzer ADVIA (120012C) was used to analyze lipid profile of IBS patients.The ADVIA Chemistry XPT System is engineered for continuous operation and timely, accurate results of samples, with an advanced user interface that is easy to use and VeriSmart® Technology to support accuracy of testing, the ADVIA Chemistry XPT System predictably and consistently delivers timely, reliable results to meet expanding workloads. Serum samples were placed inside this fully automated machine and it gives result in an hour. lipid profiling was an initial test to check lipid abnormalities in blood of IBS patients, cholesterol level HDL, LDL, VLD and triglycerides are measured for 60 our samples.

3.2.6. Vitamin D level measurement

The Adaltis EIAgen 25OH Vitamin D Total Kit is an Immuno enzymatic assay for the in vitro quantitative measurement of 25-hydroxyvitamin D2 and D3 (25OH-D2 and 25OH-D3) in human serum and plasma. This assay is intended for in vitro use only.

3.2.7.1. Principle of method

The 25-OH Vitamin D is ELISA based method. Plate has pre filled anti-Vitamin D coted wells on which calibrators control and samples are put on. A Vit-D Biotin already present in kit was also dispensed on each microwell.which compete with already present Vitamin-D in the sample, calibrator and control.next step is washing of wells, while the binded Vitamin D Biotin is measured by the Streptavidin HRP.

As concentration of HRP conjugation decreases, the level of Vitamin D in sample increases, while unbound HRP was again washed from microwell.. TMB is added and incubated, which results in the development of the blue color and stopped with the addition of stop solution. The absorbance is measured spectrophotometrically at 450nm. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The color intensity will be inversely proportional to the amount of 25-OH Vitamin D in the sample. This assay procedure run time is 100 minutes.

3.2.8. Complete blood picture of IBS subjects

The most basic analyzer from **Sysmex**, the XP-300™, hematology analyzer, performs rapid and accurate analysis of a 17-parameter CBC – Including WBC differential with an Absolute Neutrophil Count (ANC) – Histograms for RBC and WBC.

3.2.9. Transglutaminase measurement in IBS patients

Transglutaminase ELISA Kit No. ABIN511619 was used to measure level of igG in patients.

Ethical approval

The Human Ethics Committee of the Quaid-i-azam university Islamabad, approved the study proto-col. All subjects provided written informed consent.

3.3. Results

Result data is obtained by comparing severity score with other variable factors e.g age, sex, dietary pattern, physical activity.

3.3.1. Demographic and Clinical Data

Data was obtained by patients through self-administered questionnaire, dully approved by clinicians.

3.3.1.1. Age

In literature it is documented that 50% of IBS patients have symptoms of disease before they reach 35 years, and as age increases to 50 years or above IBS prevalence decreases to 50%. Age was an important parameter to check, we co-relate age with IBS to check if age has some relation with IBS progression or not. Different age groups patients were included in the study, we pick up random patients and then categorize them on basis of different age groups, starting from 10 years up-to 80 years. Highest rate of IBS was observed in age group between 31 years-40 years, and the lowest was between 71-80 years of age group, detail is shown in graph below;

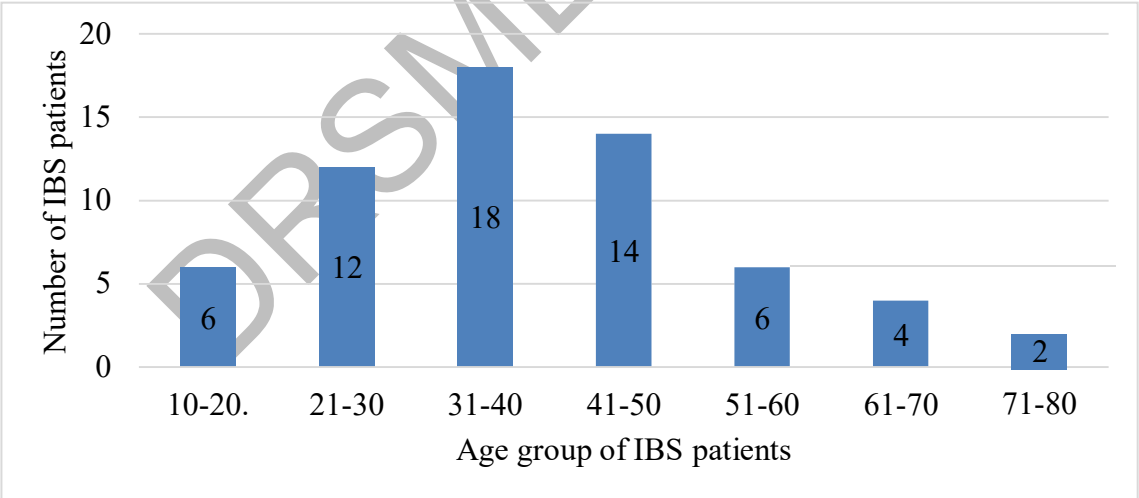


Figure 3.1: Number of IBS patients under particular age group

3.3.1.2. IBS sub types

IBS is of three sub types depending upon type of stool i.e. IBS-C, IBS-D, IBS M in IBS-M. Out of 60 patients 15 have symptoms of constipation,16 have diarrheal like symptoms while 29 patients said they have both constipation and diarrheal symptoms occasionally. Pie chart shows result:

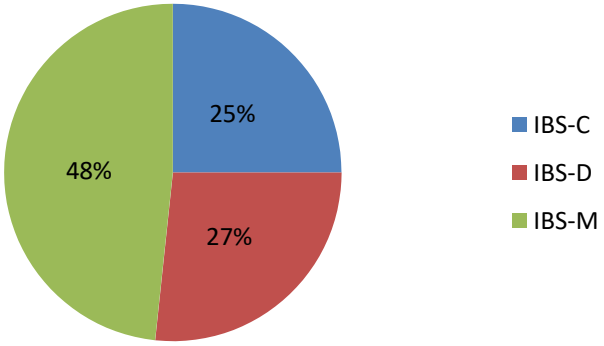


Figure 3.2: Frequency of different disease subtypes in IBS patients

3.3.1.3. IBS duration

Duration of IBS was the time period patient is suffering from this disease, this data was obtained also from questionnaire, 14 patients have IBS history for less than a year, highest number of patients were recorded between age groups 1-5 i.e. 27, 10 patients were having IBS for 6-10 years, while 4 patients have IBS for 11-15 years, some patients have a long history of IBS greater than 15 years but number of patients in this category was low i.e. 6.

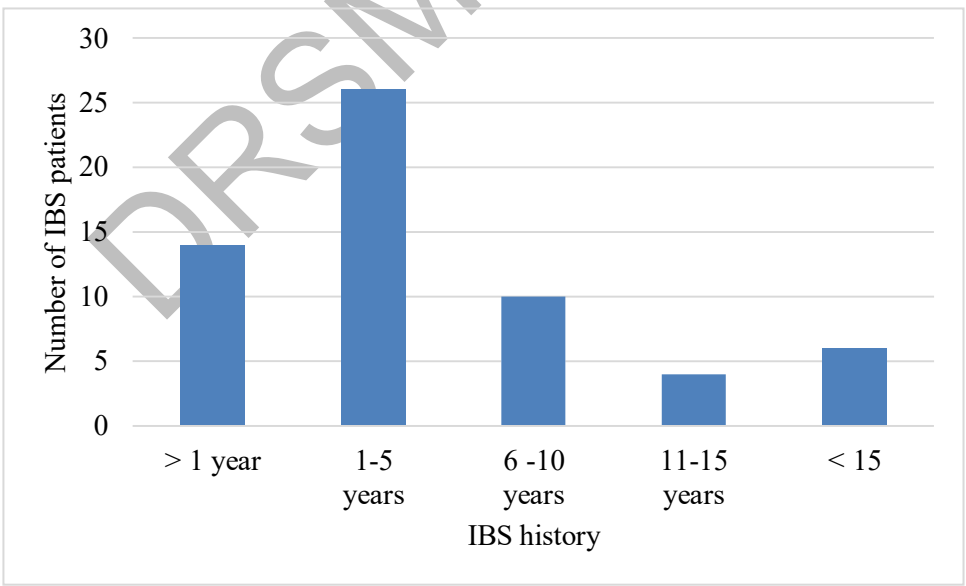


Figure 3.3: Disease history of selected IBS patients

3.3.1.6. Stress level and IBS

Stress level was measured by asking different questions, and where recorded as low, high, mild stress level in IBS patients, these are the simple questions which can easily be answered by patients (questionnaire attached at end). Results were plotted inform of pie chart: 30 patients out of 60 have stress related to IBS, while 23 have low level of stress, while 7 have mild stress history.

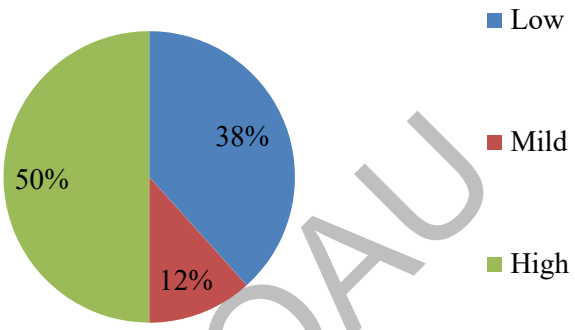


Figure 3.4: IBS Patients classification based upon stress level

3.3.1.4. Gender relation to IBS

This information was also collected using self-administrated questionnaire, this tells us about ratio of male to female suffering from IBS. Result is plotted in form of graph.

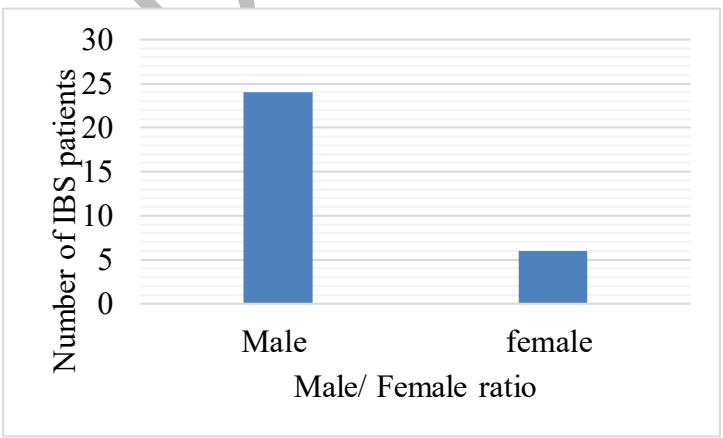


Figure 3.5: IBS patient classification on basis of gender

3.3.1.7. Physical activity and IBS

IBS can leads to reduce quality of life(QoL),In recent years there has been increased interest in evaluating QoL interventions and reports suggest the majority of IBS patients use some form of self-treatment. Studies involving healthy adults have indicated that exercise can improve feelings and symptoms of fatigue, bloating and constipation.

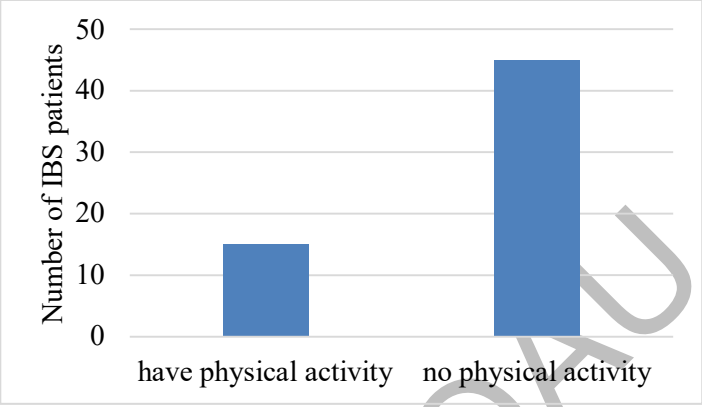


Figure 3.6: IBS patients distribution based upon physical activity

3.3.2. Analysis of Short Chain Fatty Acid

3.3.2.1. Qualitative Analysis

GC-MS was done to detect the SCFA in the serum samples the presence of butyric acid in the samples. Following are the results of two samples:

Table 3.6: GC-MS of sample IBS-9 showing the presence of butyric acid at RT 2.763 mins.

Data File	RT (min)	Peak Name	CAS Number	Area	Amt/RF	F. Match	R. Match
ID-1	2.765	Butanoic acid, methyl ester (CAS)	623-42-7	2.92E+08	0.397	922	922
ID-1	4.797	Hexanoic acid, methyl ester (CAS)	106-70-7	4.40E+08	0.598	943	943
ID-1	7.396	Octanoic acid, methyl ester	111-11-5	4.68E+08	0.636	923	926
ID-1	8.701	Nonanoic acid, methyl ester (CAS)	1731-84-6	9.89E+06	0.013	907	907

3.2.2. Quantitative Analysis

A standard curve of butyric acid was developed by using pure butyric acid in different concentrations. Butyrate peak was obtained at specific minute as shown in graph.

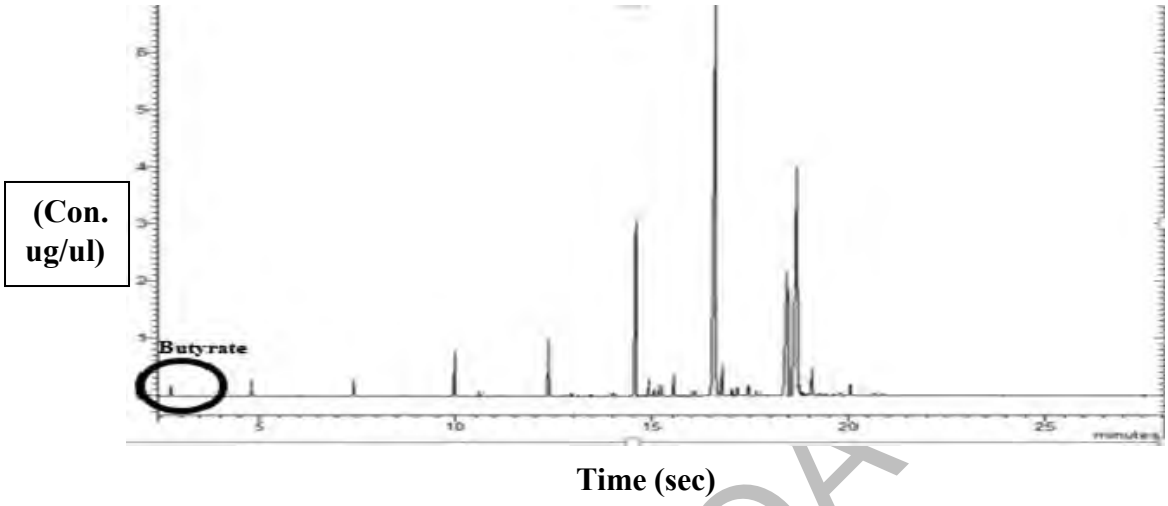


Figure 3.7: Chromatogram of IBS-9 showing peaks of different compounds present in the sample.

3.3.3 Samples of GC-FID

GC-FID of samples were run to quantitate the amount of butyric acid present in the samples. Protocol time set 10 minutes and peak at 3.7 minutes was observed for the presence of butyric acid in the samples. All the samples were run in duplicates and there mean, and the standard deviation was calculated. The following table gives a complete picture of all the samples:

Table 3.7: Concentration used for developing a standard curve and peak area obtained on GC-FID.

Sample	Concentration (Con. ug/ul)	Peak area analyzed by GC-FID (pA*s)
Butyrate	5	4380.4
	10	1.21E+04
	15	2.23E+04
	25	4.48E+04
	30	7.97E+04

Table 3.8: Mean concentration of Butyrate in IBS patients

Patient ID	Butyrate concentration uM	Butyrate Concentration Um	Mean	Standard Deviation
IBS 1	0.676865	0.768512	0.722689	0.064804
IBS 2	0.289976	0.299861	0.294919	0.00699
IBS 3	0.276576	0.287651	0.282114	0.007831
IBS 4	0.345676	0.400021	0.372849	0.038428
IBS 5	0.256784	0.234521	0.245653	0.015742
IBS 6	0.245367	0.292966	0.269167	0.033658
IBS 7	0.456784	0.345671	0.401228	0.078569
IBS 8	0.876532	0.401818	0.639175	0.335673
IBS 9	0.124567	0.127861	0.126214	0.002329
IBS 10	0.342567	0.493466	0.418017	0.106702
IBS 11	0.256785	0.234562	0.245674	0.015714
IBS 12	0.456438	0.445215	0.450827	0.007936
IBS 13	0.416786	0.421367	0.419077	0.003239
IBS 14	0.178657	0.198754	0.188706	0.014211
IBS 15	0.568796	0.567696	0.568246	0.000778
IBS 16	0.234567	0.222263	0.228415	0.0087
IBS 17	0.287652	0.284929	0.286291	0.001925
IBS 18	0.367865	0.340985	0.354425	0.019007
IBS 19	0.346782	0.328765	0.337774	0.01274
IBS 20	0.476543	0.432142	0.454343	0.031396
IBS 21	0.456721	0.432109	0.444415	0.017403
IBS 22	0.103453	0.102671	0.103062	0.000553
IBS 23	0.168754	0.173202	0.170978	0.003145
IBS 24	0.652453	0.616016	0.634235	0.025765
IBS 25	0.236786	0.326789	0.281788	0.063642
IBS 26	0.276547	0.243408	0.259978	0.023433
IBS 27	0.376541	0.320125	0.348333	0.039892
IBS 28	0.267543	0.219876	0.24371	0.033706
IBS 29	0.176543	0.178534	0.177539	0.001408
IBS 30	0.254673	0.231561	0.243117	0.016343
IBS 31	0.256743	0.258767	0.257755	0.001431
IBS 32	0.176543	0.101139	0.138841	0.053319
IBS 33	0.267865	0.297132	0.282499	0.020695
IBS 34	0.476542	0.498721	0.487632	0.015683
IBS 35	0.236572	0.213421	0.224997	0.01637
IBS 36	0.036542	0.034567	0.035555	0.001397
IBS 37	0.456432	0.435672	0.446052	0.01468
IBS 38	0.265754	0.295599	0.280677	0.021104
IBS 39	0.654321	0.526666	0.590494	0.090266
IBS 40	0.165432	0.128282	0.146857	0.026269
IBS 41	0.245742	0.201321	0.223532	0.03141

IBS 42	0.234564	0.244551	0.239558	0.007062
IBS 43	0.126541	0.121115	0.123828	0.003837
IBS 44	0.398765	0.370155	0.38446	0.02023
IBS 45	0.456231	0.492272	0.474252	0.025485
IBS 46	0.514653	0.561777	0.538215	0.033322
IBS 47	0.345682	0.398227	0.371955	0.037155
IBS 48	0.365424	0.391314	0.378369	0.018307
IBS 49	0.023476	0.021355	0.022416	0.0015
IBS 50	0.087651	0.097153	0.092402	0.006719
IBS 51	0.218765	0.292688	0.255727	0.052271
IBS 52	0.514567	0.526447	0.520507	0.0084
IBS 53	0.389765	0.336771	0.363268	0.037472
IBS 54	0.378652	0.301212	0.339932	0.054758
IBS 55	0.511193	0.500065	0.505629	0.007869
IBS 56	0.456754	0.525554	0.491154	0.048649
IBS 57	0.189765	0.160155	0.17496	0.020937
IBS 58	0.567463	0.213772	0.390618	0.250097
IBS 59	0.456432	0.503655	0.480044	0.033392
IBS 60	0.356754	0.332285	0.34452	0.017302

3.3. Lipid profiling of IBS subjects

Total 60 patients Lipid profiling was conducted, reason for this parameter study is to investigate the long-term lipid profiles of patients, Inflammatory bowel disease (IBD) which is another metabolic syndrome like IBS has been linked to an increased risk of coronary heart disease and stroke. Dyslipidemia is a well-established risk factor for cardiovascular disease. The aim of this study was to investigate the long-term lipid profiles in IBS patients and to evaluate IBS role in progression of any heart disease, because metabolic disorders are one of important cause of dyslipidemia. Sometimes IBS can lead towards inflammation due to which lipoprotein metabolism can alter leading to some variations in patient lipid profiles. In case of this study some changes are observed in IBS patients:

1. Cholesterol level decreased in 20/60 patients of IBS value ranges from 2.09-3.5.
2. 07/ 60 patients have low triglycerides (0.45-1.67).
3. Low density lipoproteins were also very low in 48/60 of IBS population, (0.45-1.99).
4. High density lipoprotein value increased in 9/60 IBS patients (2.1-4.63).
5. Very low-density lipoprotein values also show elevation in 33/60 IBS patients.

Values obtained are summarized in table from as:

Table 3.9: Lipid profiling of IBS patients

Patients ID	Cholesterol (mmol/L)	Triglycerides (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	VLDL (mmol/L)
IBS 1	5.5	4.82	1.71	1.01	3.03
IBS 2	5.7	4.48	2.1	1.51	2.02
IBS 3	4.48	5.98	2.9	1.45	3.55
IBS 4	3.2	4.46	2.9	1.04	2.24
IBS 5	2.91	3.32	1.1	1.07	1.52
IBS 6	3.47	2.91	0.7	1.15	1.32
IBS 7	6.6	4.55	2.2	1.04	2.41
IBS 8	2.5	5.71	2.3	0.95	2.66
IBS 9	3.5	4.57	1.7	1.22	2.58
IBS 10	3.5	6.42	1.2	1.21	4.38
IBS 11	3.2	6.6	0.78	1.99	4.26
IBS 12	2.8	5.87	2.53	1.45	2.82
IBS 13	3.9	4.13	2.08	0.83	2.35
IBS 14	3.6	4.88	1.22	1.31	2.11
IBS 15	2.4	5.8	2.43	0.99	3.71
IBS 16	2.5	5	2.12	1.16	2.88
IBS 17	4.5	4.07	1.3	1.18	2.3
IBS 18	3.8	5.7	2.43	1.18	3.42
IBS 19	4.08	3.47	2.16	0.85	1.68
IBS 20	2.12	4.58	1.34	1.42	2.55
IBS 21	3.5	2.145	2.3	1.56	3.3
IBS 22	5.3	3.15	0.99	1.06	1.64
IBS 23	4.3	3.65	0.89	0.95	2.3
IBS 24	3.9	3.45	1.14	0.94	1.99
IBS 25	2.09	3.93	1.67	0.96	2.21
IBS 26	2.8	2.86	0.65	1.21	1.35
IBS 27	5.3	3.71	1.59	1.13	1.86
IBS 28	5.71	4.25	0.8	1.27	2.62
IBS 29	3.8	4.56	0.76	1.45	3.2
IBS 30	3.4	5.32	0.45	1.87	2.12
IBS 31	4.4	4.56	0.41	1.34	3.2
IBS 32	3.9	2.45	0.34	0.976	1.23
IBS 33	3.4	2.98	0.55	0.87	3.09
IBS 34	2.8	3.5	0.42	0.45	2.65
IBS 35	3.9	3.5	0.34	1.43	2.34
IBS 36	3.6	2.76	0.88	2.78	3.56
IBS 37	5.8	2.43	0.99	3.71	1.1
IBS 38	3.8	2.3	0.78	2.87	0.98
IBS 39	3.93	1.67	0.96	2.21	0.76
IBS 40	4.55	2.42	1.04	2.41	1.1

IBS 41	3.44	4.3	0.45	1.34	0.54
IBS 42	3.71	1.59	1.13	1.86	0.72
IBS 43	3.47	3.16	0.85	1.68	1.44
IBS 44	3.8	3.92	0.67	1.97	2.4
IBS 45	4.07	1.3	1.18	2.3	0.59
IBS 46	3.93	1.67	0.96	2.21	0.76
IBS 47	3.8	2.98	1.09	1.9	3.45
IBS 48	3.45	1.14	0.94	1.99	0.52
IBS 49	3.2	2.78	0.54	2.45	2.5
IBS 50	4.3	0.45	0.09	1.09	3.65
IBS 51	3.15	0.99	1.06	1.64	0.45
IBS 52	2.8	0.75	1.89	1.98	1.987
IBS 53	4.13	2.08	0.83	2.35	0.95
IBS 54	3.45	1.14	0.94	1.99	0.52
IBS 55	3.3	2.09	1.45	1.89	2.56
IBS 56	4.5	1.12	1.67	1.34	1.134
IBS 57	6.42	1.82	1.21	4.38	0.83
IBS 58	4.46	2.59	1.04	2.24	1.18
IBS 59	3.2	0.86	1.09	1.54	2.54
IBS 60	2.89	0.76	1.34	3.56	0.976

3.3.4 : Correlation analysis

Severity score was developed on basis of patient's epidemiological data, and symptoms severity. Every patient was given a specific severity score, lipid profiling was compared to that severity score a weak correlation was found between severity score and lipid profiling of IBS patients. Data obtained is plotted as under for all lipid components i.e. triglycerides, LDL, HDLD, VLDL and cholesterol.

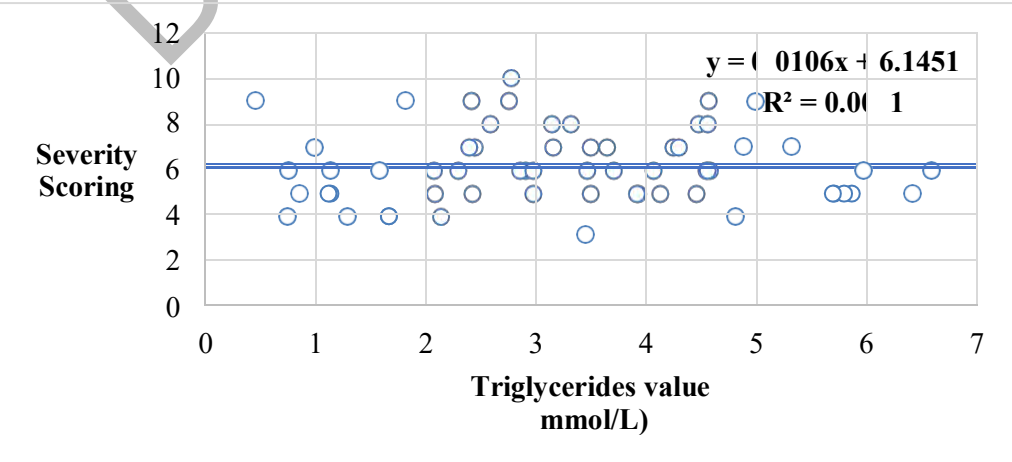


Figure 3.8: Comparing triglycerides values with severity scoring

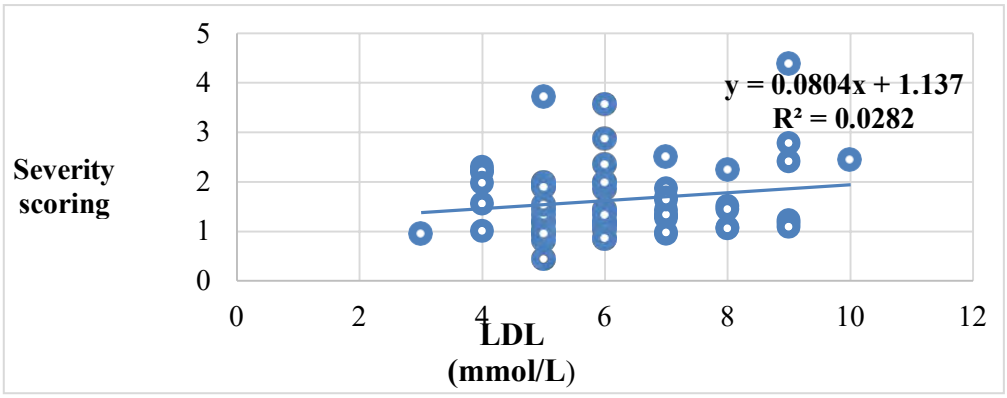


Figure 3.9: Low density lipoprotein(mmol/L) concentration compared with IBS severity scoring

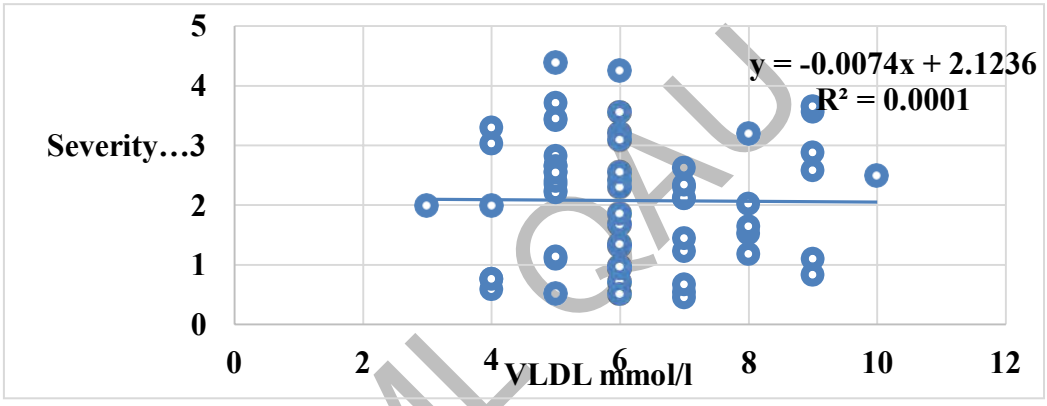


Figure 3.10: Very low density lipo protein comparison with IBS patients severity Score

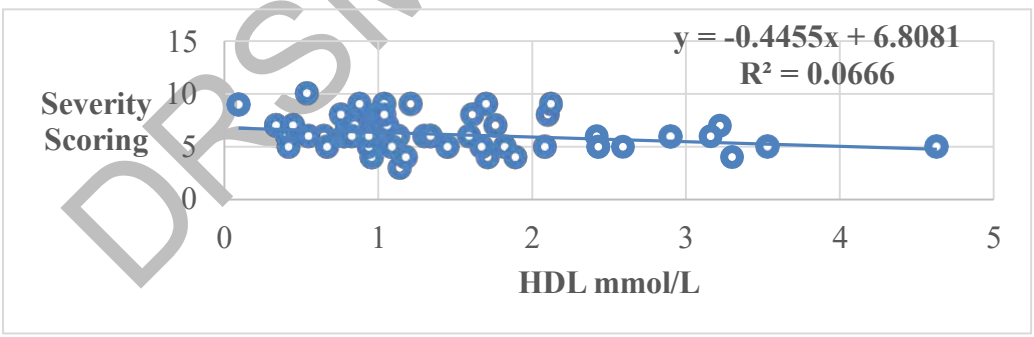


Figure 3.11: High density lipo protein comparison with IBS patient severity scoring

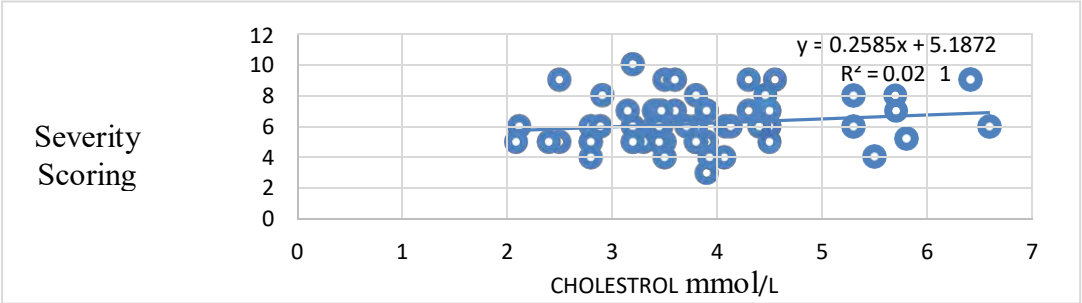


Figure 3.12: Cholesterol level comparison with IBS patient's severity scoring

3.3.4 : Vitamin D level measurement

Vitamin D may have a potential involvement in IBS pathogenesis and severity and may be in its treatment, this is what most of researchers find out until now, because vitamin D deficiency is now very common worldwide and this is related to changes in human gut microbiota which leads toward obesity, hypertension, high cholesterol, autoimmune disorders, and atherosclerotic heart disease. In our study we found some interesting results when we study vitamin D profile of irritable bowel syndrome patients. Almost all the IBS patients have lower vitamin D level, and this level is very much low if patient has a old IBS history, means more longer the duration of IBS more lower is vitamin D level. Data of all patient's vitamin D level is in following table:

3.3.4.1. Vitamin D correlation with severity score

There was a negative correlation between vitamin D level and severity score. Means as severity score increases vitamin D level decreases. IBS and vitamin D have a negative relation patient having more severe IBS has lower level of vitamin D. This can be shown as:

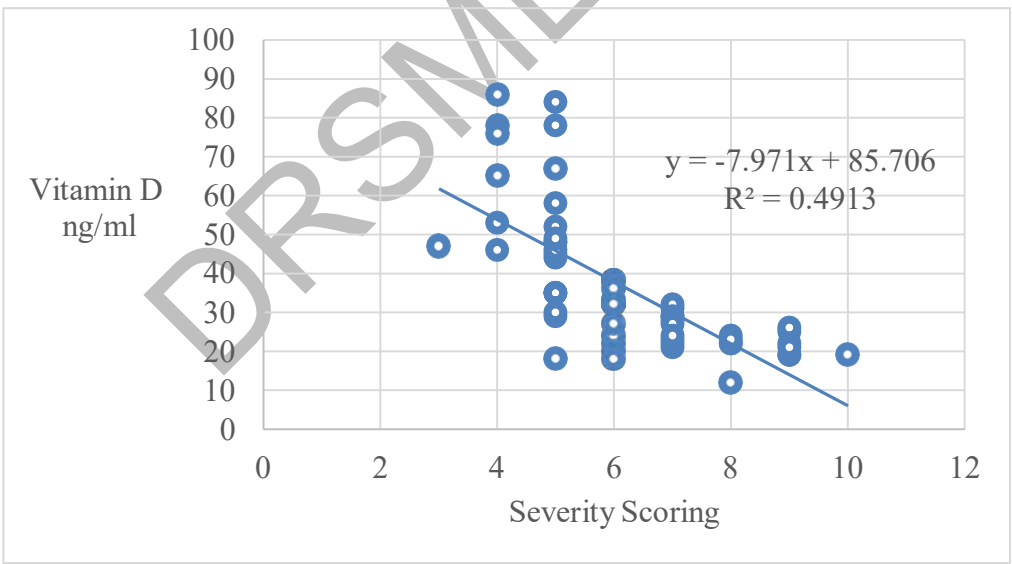


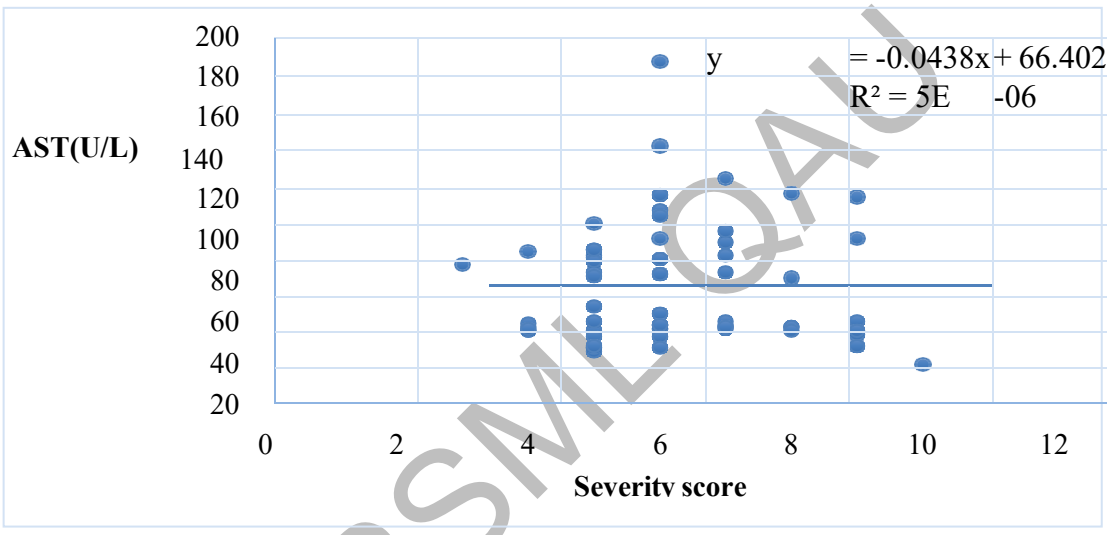
Figure 3.13: Comparison of vitamin D level with IBS patient severity score.

3.3.5. Measurement of liver enzymes

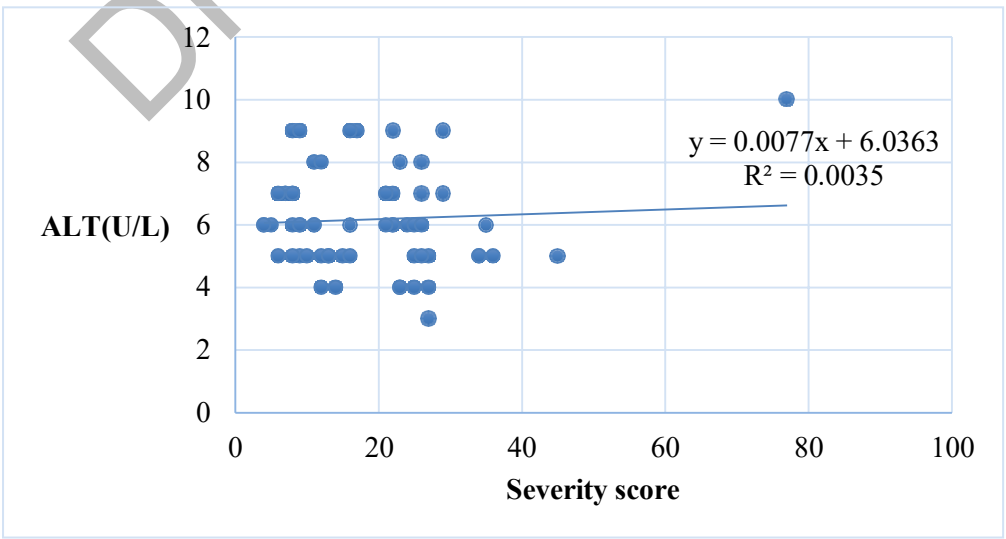
Liver enzymes measured were ALT, ALP and AST, ALT and AST were normal in majority of patients, but ALP has some variations it decreases in some IBS patients. when liver enzymes are compared with severity score no significant relationship was seen, meaning that IBS progression has no link with increases or decrease of liver enzymes.

3.3.5.1 Liver enzymes relationship with severity score

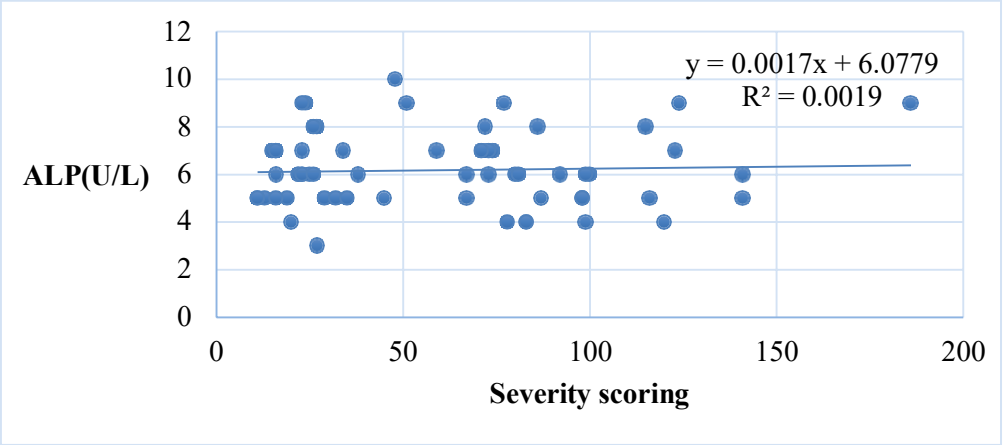
Liver enzymes correlation with severity score was done, but no significant interaction was observed with IBS progression and decrease of liver enzymes.



(a)



(b)



(c)

Figure 3. 14(a,b,c) : Severity score of IBS patients with correspondence to AST,ALT and ALP(U/L) levels in serum

AST level is almost normal in all patients, except two IBS 47 and IBS 49. ALP is low in majority of IBS patients ranging from IBS5- IBS 36 and then IBS 41, 43, 44, 45, 46, 49, 50, 51 and 56 also have lower values. ALT level is reported high in one of the patients i.e. IBS 49 while 5 IBS patients IBS 13, 19, 20, 26 and 35 have lower values.

4.6: Tissue *transglutaminase* ANTIttG test

Tissue *transglutaminase* is an enzyme helps our body in healing, people with celiac disease bodies makes such antibodies which attack this enzyme, these are called as anti-tissue transglutaminase antiboides, we performed anti ttg test to rule out celiac disease patients, all patients were negative for this test. Which confirms IBS presence along with other clinical findings.

4.7. Complete blood picture of IBS patients

Complete blood count was performed to see health status of patients or to check if patients aren't suspected to have any other health related issues, results were very satisfactory all the patients have normal blood count.

3.4. Discussion

Pathophysiological aspects of IBS were coorelated with each other. In this study, epidemiological data of 60 patients were collected; includes age, gender, IBS subtypes, type of diet, flour type, physical activity, and stress level. 31-40 years age group has maximum patients i.e. 17, then 41-50 age group have 14 IBS patients, maximum Mean

age of the patients was 39.8 years.). Major population in this study was in their twenties and below or up to 40 years. In previous studies, no correlation was observed between age and IBS, so researchers called IBS as disease of all ages because different studies proved IBS prevalence in different age groups, here are examples of some previously reported studies of IBS with relation to age groups (Drossman 2016), Alhazmi on age groups,15- 23 years; Alfetni *et al* checked prevalence of IBS of age groups 26-45 and above; and Alharthi do research on 20-59 years old(Alhaznn 2011), all studies showed presence of IBS in all age groups(Alharthi, Alhakami *et al*. 2013).

Epidemiological studies related to IBS usually shows 4:1 female to male ratio, or sometimes it can be 1:1 to 2:1 as documented by previous researches, although there is no clear study to prove prevalence of IBS is more in female then in male, scientist thought high prevalence in female is might be due to identification criteria for IBS in different population, females goes toward doctor more often than males, and they consider even minor problem more so this can be a reason why women are more diagnosed from IBS then male (Hassoun and Neuro 2012).Most of scientific reports concluded that women have more IBS symptoms than men (Arroll, Attree *et al*. 2016) having 1.5-3 folds higher symptoms of IBS were observed in them(Lee, Guthrie *et al*. 2008). Globally women have 67% prevalence then men(Lovell and Ford 2012) or symptoms normalization in these specific regions(Canavan, West *et al*. 2014).

Patients from different diet groups were included in the study and there was no effect of diet on IBS was comprehended 21 patients in this study were using whole wheat flour in their diet and 39 patients were using fine flour. 19 out of 60 patients using whole wheat flour were diagnosed with IBS-D while major 13 out of 60 patients using fine flour were diagnosed by IBS-C. Insoluble fiber in the whole is considered to worsen the symptoms and give gluten intolerance type symptoms in IBS patients (Guo, Niu *et al*. 2014). Sometimes symptoms of IBS varies according to food item they consume like milk and milk products, wheat products, cabbage, onion, peas/beans, hot spices, and fried food(El-Salhy, Mazzawi *et al*. 2016), but scientist doesn't observe any difference between IBS and normal persons food intake choice(Østgaard, Hausken *et al*. 2012).Some previously findings explained food allergies/intolerance can be related to IBS but diet correlation with IBS is yet not understood completely(El-Salhy, Hatlebakk *et al*. 2015).

fortysix out of sixty patients selected had no physical activity while 10 patient has moderate level physical activity, lack of physical activity can worsen IBS symptoms, and longtime inactiveness can lead toward IBS . Johannesson *et al*. showed that an increase in the physical activity helps soothing of IBS symptoms and improving quality of life in IBS patients (Johannesson, Ringström *et al*. 2015). Life style changing from low physical activity to have more physical activity can sometimes improve symptoms of IBS, so this can be used as a treatment procedure along with other treatment methods(Harris and Roberts 2008).

Among IBS subtypes, IBS-M is most prevalent, and IBS-C, IBS-D ratio is almost same, one of the study conducted on comparison of male and female regarding IBS subtypes concluded that IBS-M was most prevalent among male population systematic review of population-based studies, the prevalence, while another study showed not different in men and women (25.0 vs. 25.8%,respectively) in IBS-M subtype symptoms.(Masud, Hasan *et al*. 2001), reported 56% of IBS patients fall in category of mixed subtype IBS, while Constipation-predominant and diarrhea-predomi-nant IBS were reported in 17 and 27%, respectively, another finding conforms with the studyby Masud *et al*. in Bangladesh,10 which reported the prevalence of diarrhea-pre-dominant IBS, constipation-predominant IBS, and non-specific IBS as 19, 0.8, 16 and 64%, respectively (Rajendra and Alahuddin 2004).

Psychosocial stress likely alters gut microbiota, increases mucosal permeability, motility, and induces visceral hyperalgesia in IBS patients (Moloney, Johnson *et al*. 2016).out of 60 patients included in study 50% have high stress, while 23% are not having any symptoms of stress, 7% patients have a mild stress, some of the previous studies related IBS to induce stress in patients, like in a 12 year prospective study patients with functional GI disorders including IBS are more likely to have depression and anxiety, anxiety and depression were major risk factor for developing IBS.(Koloski, Jones *et al*. 2012).So scientist believe relation between IBS and psychological distress is a two way process, one can initiate other if stay for longer(Spiller, Aziz *et al*. 2007). Another study indicated presence of anxiety in majority of IBS patients, and proposed that IBS patients are more likely to get anxiety and depression than way around(Koloski, Jones *et al*. 2016, Von Wulffen, Talley *et al*. 2019).Similarly (Tanaka, Kanazawa *et al*. 2018), note high level of psychological stress in IBS patients, in there experiment IBS patients showed

high level of colonial motility in response to experimentally induced stress when compared to healthy groups.

Quality of life is reduced in IBS patients, most of the patients i.e. 27 were suffering from 1-5 years, and 14 were suffering from IBS from last year, IBS symptoms are periodic they often recur and disappear impetuously.

Human gut has the ability to absorb vitamin D especially in ileum approximately 70-80% absorbed here (Sprake, Grant, & Corfe, 2012). Human gut also contains receptors and regulatory mechanism for vitamin D which are mainly located in cecum and colon regions, many studies have been done so far to check correlation of vitamin D level and IBS patients, so it's important to look for vitamin D deficiency in IBS patients.(Vestergaard, 2003). (Nwosu, Maranda, & Candela, 2017b) corelate vitamin D and IBS, in which they found significantly lower level of vitamin D in IBS patients i.e. 25 (OH) D: 53.2 ± 15.8 nmol/L compare with control group 65.2 ± 28.0 nmol/L. lower level of vitamin D in IBS patients may be due to combination of factors e.g. food to be eaten , way of living, time of exposure to sunshine, or may be due to some medical reason like lower level of albumin in blood(hypoalbuminemia).(Khayyat & Attar, 2015) check frequency of vitamin D in IBS patients and concluded that vitamin D deficiency was very high in IBS patients(82%), so a significant negative correlation exist between IBS and vitamin D. These studies are in accordance with our result which also showed a significant negative correlation between IBS and vitamin D level, all IBS patients have low level of vitamin D.

SCFA are the fermented by-product of bacterial metabolism in the human gut. Acetate, propionate, and butyrate are the three major SCFAs detected in the gut. In this study, we used a manual protocol for the extraction of SCFAs from the serum (Skoglund, 2016)We are successful in the extraction of butyrate from the serums. Acetate and propionate cannot be extracted, may be due to some differences in the environmental factors. Butyrate is considered as the holy grail for gut growth and development due to its direct effect on the colonocytes and its anti-inflammatory properties. Farup *et al*. showed that the production of butyrate decreases during the curse of IBS (Farup *et al*., 2016)Level of butyrate extracted is low. This may be due to the factor that it has been extracted from serum. IBS patients have low SCFAs production in our result, which is in accordance with previous research work. Cumming *et al.,* showed the level of SCFAs measured in

the portal, hepatic and peripheral were in highest 24 μmol/l in the portal, 12 μmol/l in the hepatic and lowest 4 μmol/l in the peripheral (Gargari *et al*., 2018).

The association between IBS and the gut microbiota has been demonstrated in several studies and efforts have been made to characterize the abnormal microbiota in patients with IBS (Bennet *et al*., 2015) So far, the results have been in consistent. For the human health and disease, the function of the microbiota might, therefore, be as significant as the phylotype. It has been argued that"the phylotype provides the environmentally selected inter-face for the functions(Avershina & Rudi, 2013). The function could be measured as chemicals and metabolites in the faeces. The microbiota metabolises non-digestible food constituents into short-chain fatty acids (SCFA) that have extensive immunological and regulatory functions and appear to be the link in the host-microbe interactions (Furusawa *et al*., 2013).

Studies are now focusing on studing a close association between gut microbial changes and alteration of metabolic pathways in gut. It is now confirmed that these gut changes can leads to hepatic injury and disorders, one of common example in insulain resistance by gut microbiota (Gerritsen, Smidt, Rijkers, & de Vos, 2011). Along with these metabolic disorders microbiota changes are also reason of causing functional gastrointestinal disorders (FGIDs).

Amall intestinal bacterial overgrowth is also due to alteration of gut normal microbiota, and finally leads to IBS (Z. Liu, Que, Ning, Wang, & Peng, 2013), intestinal permeabilty is very common among these disorders, which also effect hepatic disfunctioing. Liver enzymes ALT/AST/ALP also got eleveated due to this condition (Pande, Kumar, & Sarin, 2009), (Park *et al*., 2009), (Cortez-Pinto, de Moura, & Day, 2006) Thus, it is possible that IBS itself could cause increase in liver enzymes. In patients having hepatitis IBS is responsible for elevation of gut permeability and endotoxin production which also produce tumor necrosis factor-alpha. (Guo *et al*., 2014).

Very few epidemiological studies have evaluated the association between IBS status and metabolic syndromes, invitro studies can explain role of lipid lowering effect of intestinal bacteria like some strains of lactobacillus and bifidobacterium(Sabaté *et al*., 2008).

to conclude this phase we can say, Irritable bowel syndrome (IBS) is an anomaly of gut microbiota leading to symptoms like flatulence, abdominal cramps, and pain. Gut microbiota produces short chain fatty acid by fermentation of non-digestible carbohydrates. In IBS, change in the diversity of gut microbial community leads to altered levels of SCFA production. In this study, it was found that the gender of the patients, type of flour and diet has no effect on the prevalence of IBS while physical activity greatly cures symptoms related to the IBS. Blood profiling of IBS patients showed that a high level of triglycerides is associated with the prevalence of IBS

Chapter 4: Gut Microbial diversity and correlation with IBS.

4.1. Introduction

4.1.1 Intestinal flora of human

Complex microbial community resides inside human intestine, trillion of microbes as a part of that complex system have immense effect on host physiology and metabolic activities .(Catanzaro *et al*., 2014). Microorganism present resides in a balance, and in their normal balance they are not harmful to host but any disturbance to this normal microflora leads to disease condition. Irritable bowel syndrome is a disease of large intestine and now many scientists believe that its due to disturbance in normal microflora of intestine (Zhou *et al*., 2016).

Microbial community colonized inside the human body is mutually named as microbiota. Highest number of microorganisms are present in human colon, nearly 1013-1014, out of which most are bacteria(Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012a). These normal gut microbiota has a very positive role in maintaining host health's, as it protects from pathogens to grow there(Fukuda *et al*., 2011) more over it also play role in host physiology like nutrients absorption from daily diet taken, (Yatsunenko *et al*., 2012b), also they maintains host normal immune functions(Yatsunenko *et al*., 2012b). So changing in normal gut microbiota is a factor leading toward multiple disease onset mainly metabolic disorders like obesity type 2 diabetes mellitus (DM2), (Turnbaugh, Bäckhed, Fulton, & Gordon, 2008), as well as gastric problem (Yamaguchi *et al*., 2016).

4.1.2. Study on changing intestinal flora

4.1.2.1. Culture dependent techniques

Study of gut microbial changes is an important factor to understand role of these in health and disease, certain culture-based analysis on fecal microbiota of IBS patients reveals decrease amounts of Bifidobacteria and Lactobacilli, although culture based study of microbiota is cheap and easy to perform, it doesn't give us a true picture of gut, so recently some new techniques are emerged which give scientist a better insight to study gastric problems like IBS, culturomics is one of such technique, basically this is a culturing technique that utilizes multiple culturing conditions and then matrix-assisted laser desorption/ionization-time of flight, recently 16S rRNA for microbial identification is more powerful tool of study for microbial diversity inside gut(Lagier *et al*., 2016). But

much less data is recently there using this technique to analyze intestinal flora of irritable bowel syndrome patients (Lagier *et al*., 2016).

4.1.2.1. Culture independent techniques

Culture independent techniques are now accepted as more powerful and wide ranging for observing and studying changes in normal microbiota of IBS patients (Hayashi, Sakamoto, & Benno, 2002).Among these molecular based techniques quantitative polymerase chain reaction (qPCR) and 16S rRNA are most popular due to their accuracy.

Although many gut microorganism are not possible to culture with traditional culturing methods its still considered as gold standard for detection and classification. New culture independent techniques are now based on 16s RNA characterization of microbial community, due to which its now possible to understand microbial community interactions and role in IBS patients. Classical 16s RNA techniques like fingerprinting Denaturing gradient gel electrophoresis (DGGE), Terminal restriction fragment length polymorphism (**TRFLP** or sometimes **T-RFLP**) and polymerase chain reaction PCR which were once very reliable for gene amplification are now paired with high throughput 16s RNA techniques , which leads to more accurate and reliable observation of gene study, and by using these combinations thousands of genes are identified and quantified with a very short span of time(Simrén *et al*., 2013).

One of the techniques used now a days to analyses gene of interest in microbial phylogenies is marker gene analysis, usually marker gene sequencing uses primers that target a specific region of a gene of interest in order to determine microbial phylogenies of a sample. Marker gene contains a region that is highly variable and can help for detailed analysis of conserved region, this act as a binding site for PCR primers. Amplification and sequencing using marker gene are now considered to be more cost effective and fast then other methods for a general overview of microbial ecosystem inside gut (Walker *et al*., 2015).

One of the other techniques of studying gut microbial gene is using whole metagenome analysis, which helps in identification of all genes present in sample. Although compared to other techniques metagenomic analysis gives more information and phylogenetic analysis diversity but this is much expensive than others. This method captures all DNA either its bacterial, viral or eukaryotic. Whole metagenome not only give large number of

sequencing depth but also taxonomical analysis up to species or strain level (Scholz *et al*., 2016). This technique also tells about functional capacity of a micrbial community at gene level, (Abubucker *et al*., 2012).

4.1.3. Gut microbiota of IBS patients

Scientist believe that fecal microbiota is a representative of intestinal microflora so analysis of fecal microbiota of IBS patients give a picture of intestinal flora of patients. Studies are there which proved a lower diversity of fecal microbial in IBS patients(Noor *et al*., 2010). Phylogenetic diversity also varies among IBS and healthy individuals, IBS patients have lower phylogenetic diversity compared to healthy individual(Jeffery *et al*., 2012).

Beside fecal, mucosa-adherent microbial community also have role in IBS progression, some of report highlighted changes in microbiota of both gut as well as mucosa adherent microbial community in IBS patients(Carroll, Chang, Park, Sartor, & Ringel, 2010).One of the study carried out by (Kerckhoffs *et al*., 2009) showed higher prevalence of Pseudomonas aeruginosa in both fecal and musical samples of IBS patients.

Many research groups are now focusing on gut microbial identification of IBS patients, mainly by using culture independent techniques(Zoetendal, Rajilić-Stojanović, & De Vos, 2008), and these studies clearly reveal altered microbiota of IBS patients compared to healthy individuals (Carroll *et al*., 2011). Most of studies showed enrichment of gut microbial composition with Firmicutes and lowered number of Bacteroidetes in IBS patients, compared to healthy ones(Rajilić–Stojanović *et al*., 2011).

Gut microbiota of Irritable Bowel Syndrome patients showed changing in normal microflora of patients, Malinen *et al*(Malinen *et al*., 2005) were the first group of scientist to study these changes deeply according to them amount of *Bifidobacterium catenulatum* and *Clostridium coccoides* in stool sample of IBS patient was very low as compared to healthy individuals. One of other important finding in their work was amount of lactobacilli calculation, they found that in IBS-Diarrheal patients' low amount of lactobacillus were present as compared to IBS-Constipation. Similarly, Veillonella spp number was very high in IBS-Constipation patients than healthy controls. This was the first-time scientist used high throughput 16S rRNA gene cloning and sequencing to study fecal microbiota of IBS patients, and they found differences in intestinal

microbiota of IBS patients especially in phyla Firmicutes and Actinobacteria (Malinen *et al*., 2005).

IBS symptoms are corelated to type of bacteria present in gut, a positive correlation is there between intestinal symptoms and gamma proteobacteria quantity in gut while Bifidobacterium and IBS symptoms are negatively corelated(Rajilić–Stojanović *et al*., 2011). Cyanobacteria presence in IBS patients is associated with increase gastric symptoms like pain and blotting, while proteobacteria high level in IBS patients increases symptoms of mental stress and pain threshold frequency(Jeffery *et al*., 2012). Similarly, Actinomycetes order having family Actinomycetaceae are also known to have increase stress induction in IBS patients. As number of bifidobacterial and lactobacilli decreases in IBS patient there frequency of passing stool increases per day (G. Parkes *et al*., 2012).More studies are needed to clear relationship between gut microbiota and onset of IBS symptoms, which will help in understanding pathogeny of IBS(De Filippo *et al*., 2010).

4.1.4. Bioinformatics tools for 16s rRNA sequencing data

QIIME (Quantitative Insights Into Microbial Ecology) a bioinformatics pipeline, which analyze data generated by marker gene or amplicon sequencing(Caporaso, Kuczynski, *et al*., 2010). It starts from quality control analysis, leading toward phylogenetic level doing clustering analysis as well. It can do OTUs formation, sequence variants and taxonomically annotation of data(Kuczynski *et al*., 2011). One of main feature of QIIME is formation of feature table, which not only describes abundance of each OTUs but also reveals sequence variants. In addition to this QIIME also help in determination of rarefaction, alpha diversity and beta diversity calculations, visualizations such as principle coordinates analysis (PCoA) and many other. This was first released in 2009(Caporaso, Kuczynski, *et al*., 2010), and now is updated as QIIME 2 in 2019(Bolyen *et al*., 2018). We used this software for analysis of our results.

Another software used in result discussion was COSMOS ID, which has potential to provide rapid and reliable tools for 16s amplicon bacterial detection.

4.2. Aim and Objectives

This study aim was to check different type of bacteria present in gut of IBS patients that were isolated and selected on basis of chapter 3 study,using 16S rDNA technique, objectives of study were:

1. Assessment of different microbial phylum which are more prevalent in IBS patients.
2. Corelating metadata with microbes present, which will help in making connections between symptoms onset and disease progression.

4.3: Material and methods

4.3.1. Experimental design

It's a cross sectional study. A total of thirty Irritable bowel syndrome patients(according to WHO formula, link already given) were selected for this study. All patients were recruited according to inclusion and exclusion criteria designed for this study. Stool sample was collected from each patient and stored at -80 till further process. Stool samples were then subjected to culture independent microbiological analysis.

4.3.2. Metagenomic analysis of IBS Stool samples

The impact of gut microbial diversity of IBS patients on progression of disease was assessed by metagenomic analysis. Based on altered blood parameters, disease symptoms severity and availability of samples 30 samples were selected for this metagenomic study.

4.3.2.1. DNA extraction

Favor prep Stool DNA isolation mini kit (Favorgen) was used for metagenomic DNA extraction according to manufacturer instructions.

4.3.2.2. Gel Electrophoresis

Gel electrophoresis was carried out to confirm DNA after extraction. For this 1% agarose gel was prepared.

4.3.2.3. DNA quantification using Nano drop

Thermo Scientific Nano Drop 1000 spectrophotometer was used to check quantity of DNA extracted at 260 nm wavelength. 1µl of illusion buffer was used as blank. Samples were run after blank using micropipette, and results were recorded.

4.3.3. Metagenomic analysis

4.3.4.1 PCR Amplification, *Illumina MiSeq* Sequencing and Data Processing

DNA extracted from 30 samples of IBS patients were subjected to Illumina MiSeq sequencing at UMGC (University of Minnesota Genomic Center). Genome sequencing included following steps:

- Isolation
- Purification
- Fragmentation
- Ligation to adopters
- Purification.

4.3.4.1.1 Amplicon sequencing

V4 region of 16S rRNA gene was targeted for bacterial species, using universal primers: 515f(5'-GTGCCAGCMGCCGCGGTAA-3')

806r(5'-GGACTACHVGGGTWTCTAAT-3') (Gohl *et al*., 2016).

Illumina MiSeq plant form using 2×300-bp paired end protocol, was followed for sequencing.

4.3.4.1.2 Bioinformatics

Following steps are involved in data processing:

1. Data was analyzed using QIIME v.1.8.0 (Caporaso, Bittinger, *et al*., 2010).
2. Adapter removal of low-quality regions (< Q30) were removed using Trimmomatic v. 3.2 (Goldman *et al*., 2006).
3. Some reads are less or greater than 50 bases of total amplicon length were also discarded.
4. Panda seq was formed using fastq join scripts which make joining of high-qualityreads ((Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012)).
5. Chimeras formed, were identified using UCHIME v. 6.1(Edgar, 2010).
6. A confidence score of 80% bootstrap of RDP training v. 9 was used for classification of sequences against a naïve Bayesian(W. Wang, Scali, Vignani, & Mazzuca, 2003).

7. UCLUST was used for clustering of Open-reference operational taxonomic units (OTUs) at 3% dissimilarity
8. A comparison analysis was done using SILVA v.132 16S rRNA database at PyNast (Caporaso, Kuczynski, *et al*., 2010) (Edgar, 2010);(Quast *et al*.).
9. Further statistical analysis was done using OUT count rarefied to 10,000 sequences per sample.
10. Good's coverage, Chao1, Shannon, and Simpson's E indices were used to calculate alpha diversity.
11. Principal coordinate analysis (PCoA) was calculated using Bray-Curtis dissimilarity matrices.
12. Further Statistical data analysis was done using CosmosID Metagenomics Cloud which not only helps in analyzing microbial diversity, but also give a comparison anlysis of all variable factors with 16S rRNA data.

Data was analyzed using QIIME2 software **(Bolyen *et al.*, 2018),** it includes following steps:

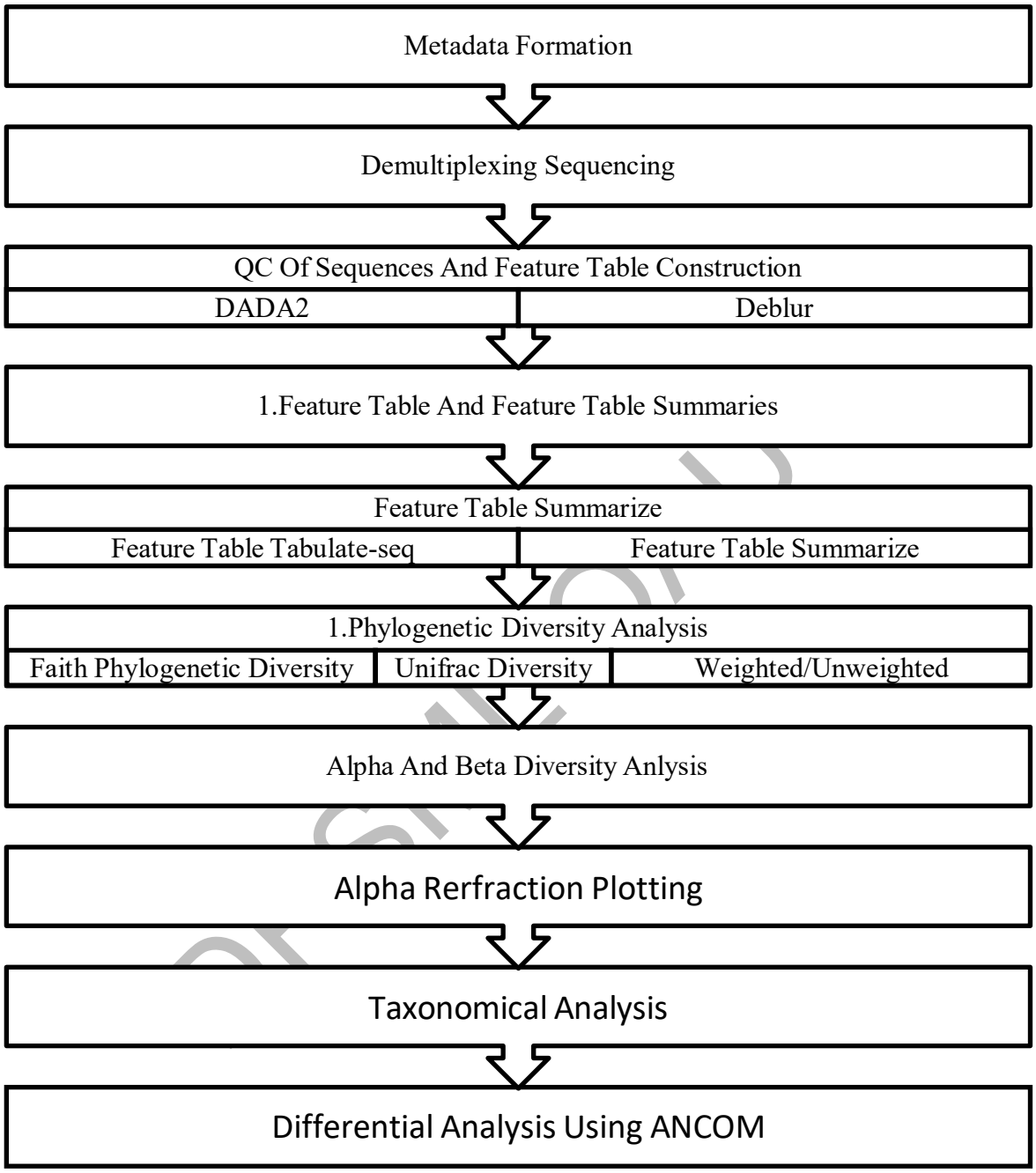


Figure 4.1: Flow chart of result analysis.

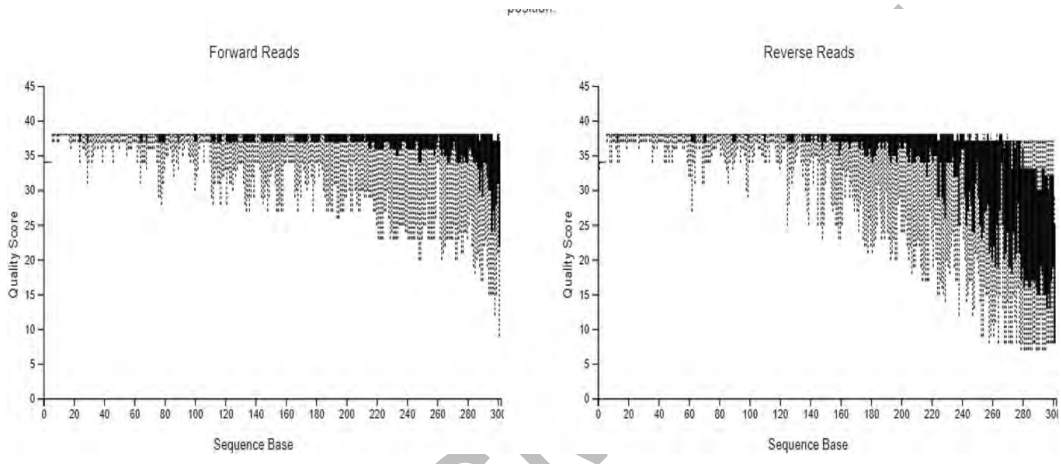
4.4. Results

4.4.1 Meta data formation

The data was obtained from Illumina Miseq(model) plat form, first step was formation of metadata in google sheath and demultiplexing of samples. Meta-data is attached as annexure.

4.4.2 Demultiplexing sequences

In-order to reduce probabilities of sequences errors and dereplicating sequences, reads must be demultiplexed, which was used to generate OTU.



(a) **(b)**

Figure 4.2: (a) Forward reads plots were made by using random sampling of 10000/1573417 sequences without any replacement, 301 bases were identified that have minimum sequence length**.** We created reverse plot same as that offorward reads i.e., using 10000/1573417 random sampling without any replacement, during subsampling minimum sequence length was 301 bases.

4.4.3 QC of sequences and feature table formation

Feature table is measure of number of features repeated for each sample in data set. QC was determined using two deblur. Result is represented in table.

Table 4.1: QC of sequences and feature table construction using Deblur analysis tool

S.nu	Fraction-artifact- with minimize	Fraction-artifact	Reads-derep	Unique-reads-deblur	Reads-deblur	Unique- reads- hit-artifact
IBS22	1112	0.857914	158	41	76	2
IBS20	2272	0.660211	772	116	248	18
IBS16	21716	0.638101	7859	284	1223	28
IBS21	12499	0.610849	4864	361	1041	80
IBS17	40858	0.591439	16693	761	2827	137
IBS15	46593	0.588694	19166	815	2998	142
IBS24	46005	0.563026	20103	597	3029	93
IBS19	57466	0.552553	25713	711	3703	135
IBS23	44062	0.526894	20846	737	3470	147
IBS26	50802	0.524901	24136	863	3920	174
IBS18	59610	0.520081	28608	1208	4986	214
IBS13	41579	0.504509	20602	501	2935	80
IBS30	54951	0.492584	27883	1192	4783	228
IBS25	57944	0.487177	29715	628	3738	130
IBS27	57339	0.479412	29850	595	3723	108
IBS09	66030	0.462562	35487	1886	6208	399
IBS28	57977	0.453007	31713	732	4582	152
IBS06	74991	0.438373	42117	1125	5885	228
IBS07	96588	0.419069	56111	2173	9001	484
IBS11	72850	0.388099	44577	1148	6610	230
IBS14	49515	0.349813	32194	147	3968	29
IBS08	0.68326	0.34855	44511	774	5726	129
IBS29	45787	0.341844	30135	516	4479	89
IBS02	21225	0.340966	13988	534	2295	98
IBS10	64644	0.315528	44247	945	6426	197
IBS12	58397	0.291522	41373	254	5672	59
IBS04	81405	0.269836	59439	675	9123	132
IBS01	59042	0.26432	43436	231	5390	32
IBS03	77607	0.257503	57623	488	6612	81
IBS05	84225	0 192425	68018	84	8777	5

4.4.4 Feature table summary

Feature table was formed after denoising and clustering, showing frequencies of all variables compared with sequences

Table : 4.2 Feature table showing summary of features per each IBS patients.

Patient ID	Frequency	Patient ID	Frequency
IBS05	8636	IBS30	3013
IBS04	7788	IBS27	2835
IBS03	6038	IBS25	2751
IBS12	5144	IBS19	2721
IBS01	5067	IBS26	2650
IBS07	4978	IBS23	2368
IBS10	4872	IBS24	2342
IBS08	4569	IBS13	2270
IBS11	4526	IBS17	1947
IBS06	4215	IBS15	1850
IBS29	3677	IBS02	1160
IBS14	3669	IBS16	937
IBS28	3470	IBS21	621
IBS18	3165	IBS20	101
IBS09	3017	IBS22	28

4.4.5 Phylogenetic diversity analysis

4.4.5.1. Alpha rarefaction of microbiome NGS data of samples from IBS patients

Alpha rarefaction statistical tool is used to compare alpha diversity as a function of sampling depth. By the QIIME2 diversity alpha-rarefaction visualizer we obtain following results.

- Shannon's diversity index which gives quantitative measure of community richness in IBS patients stool sample.
- Observed OTUs (a qualitative measure of community richness) in IBS samples.
- Faith's Phylogenetic Diversity, that is qualitative measurement of feature table and phylogenetic relationship.

Shannon's diversity observed OTU and faith's phylogenetic diversity were compared with metadata column having categorical data. Categorical data includes bar code, IBS patients sub type, vitamin D level, flour type, depression level and other GIT disease compared with three levels of alpha rarefactions.

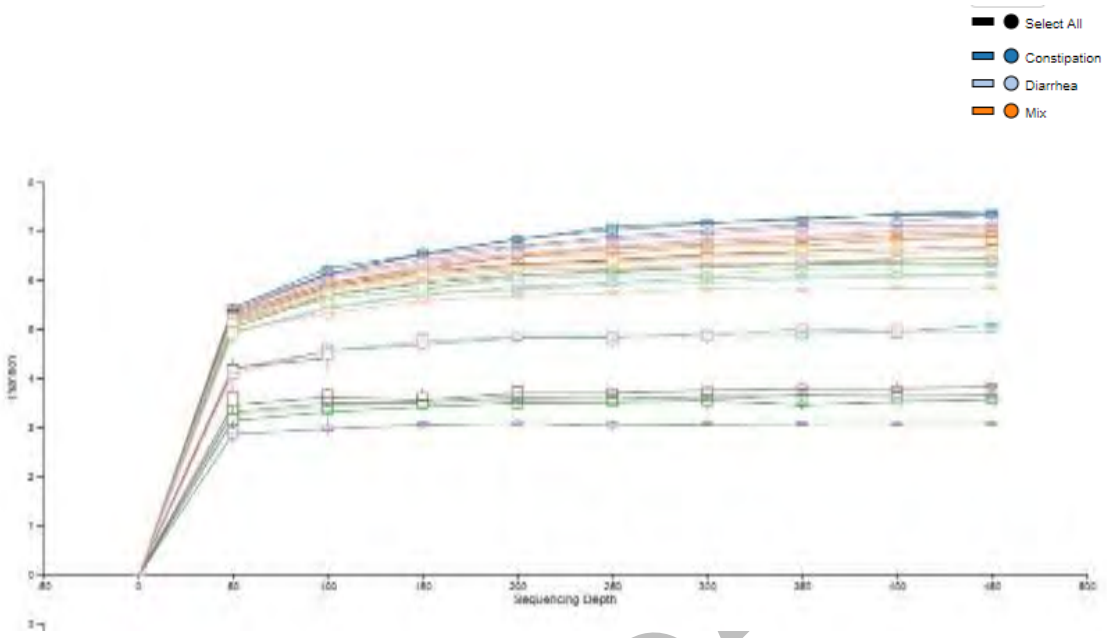


Figure 4.3: Shannon's diversity vs sequencing depth of barcodes by metric observed OTU

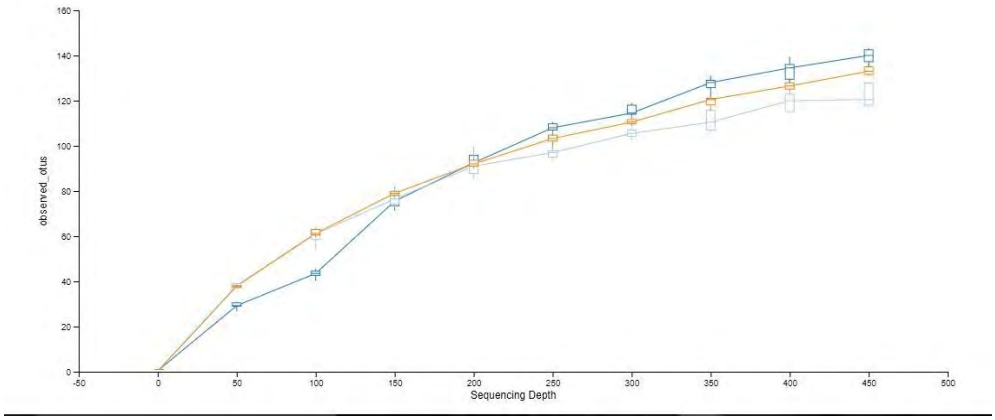


Figure 4.4: Shannon's diversity vs sequencing depth of IBS types by metric observed OTU

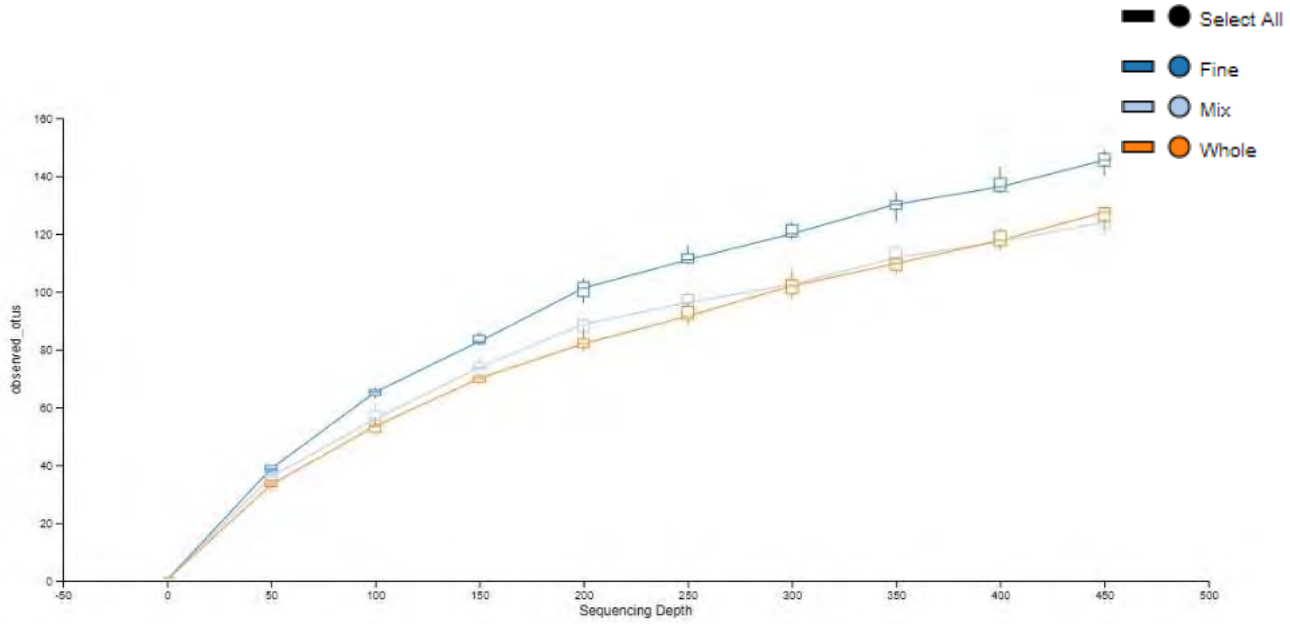


Figure 4.5: Shannon's diversity vs sequencing depth of flour consumption in IBS patients by metric observed OTU

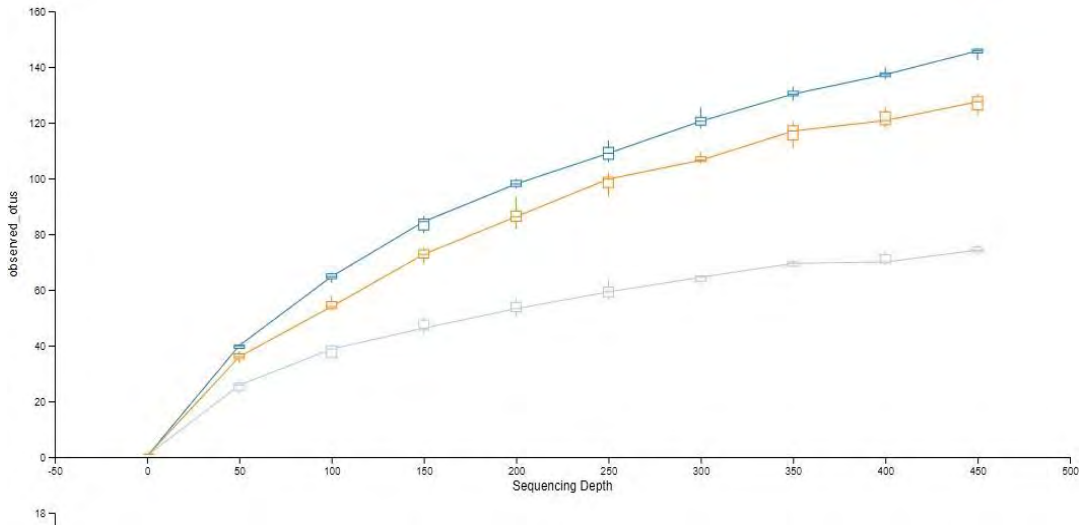


Figure 4.6: Shannon's diversity vs sequencing depth of depression level by metric observed OUT

4.4.5.2 Differential diversity analysis of microbiome in IBS patients' samples: beta diversity

Relative abundance of sequence reads was analyzed using PCoA, As recommended by Navas-Molina *et al*., (Navas-Molina *et al*., 2013) analyzing the presence of clustering and grouping in data principal coordinate analysis (PCoA) was used. Which not only reveals information about variation in data but also let to check similarities and difference among samples. Beta diversity analyses were performed, which help in estimation of phylogenetic estimation of community similarities.

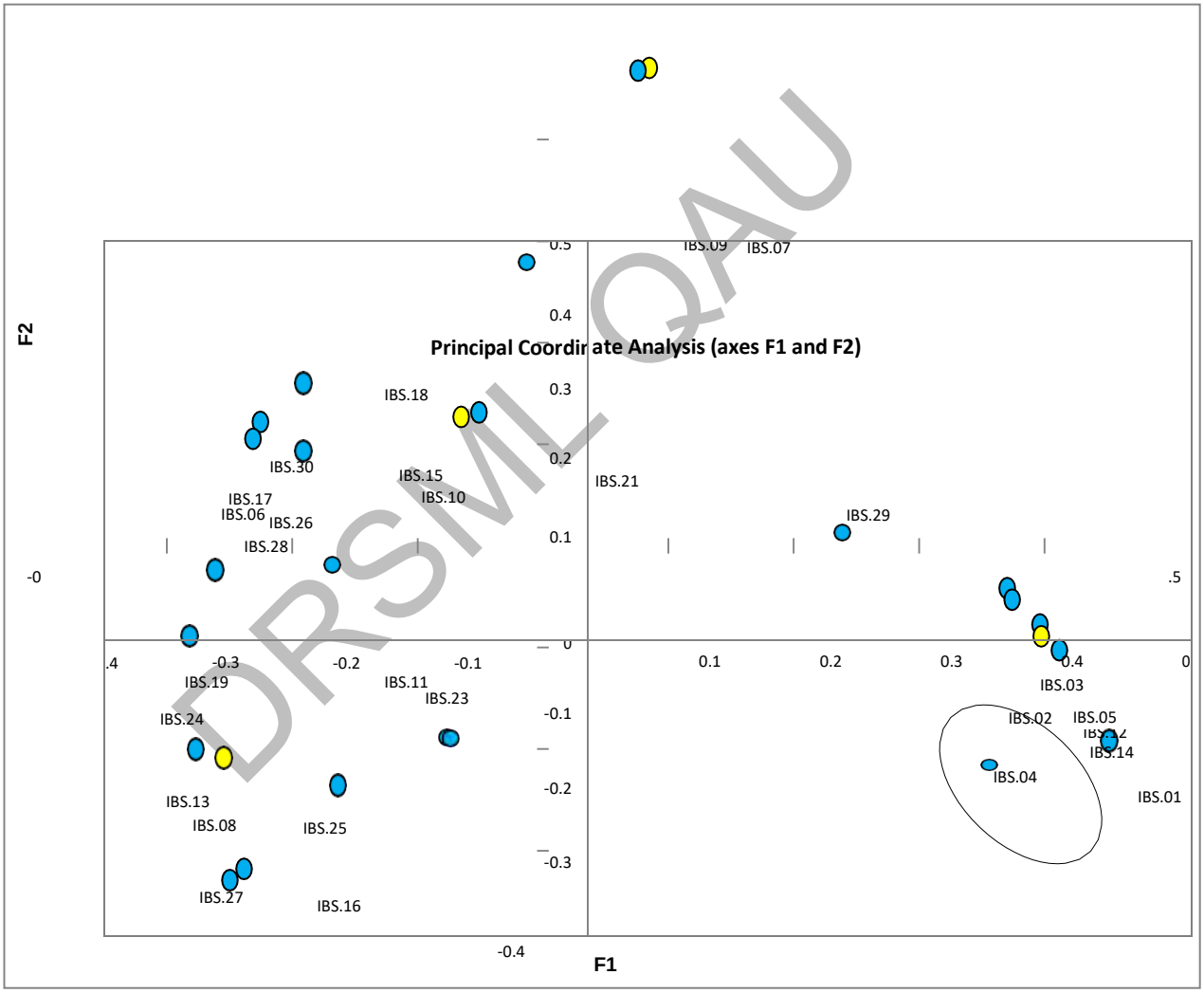


Figure 4.7: Principal coordinate analysis (PCoA) analyzing diversity among IBS patients

4.4.5.3 Relative abundance of bacterial phyla in IBS patients:

30 enrolled patient's data were analyzed to check relative abundance of bacterial phyla. Among taxonomical (phylum level) analysis 36 phyla were observed, that are: Epsilonbacteraeota, Gemmatimonadetes, Ignavibacteriae, Brc1, Fibrobacteres, Bacteroidetes, Fusobacteria, Armatimonadetes, Tenericutes, Nitrospirae, Synergistetes, Crenarchaeota, Firmicutes, Gracilibacteria, Planctomycetes, Actinobacteria, Ws1, Omnitrophica, Bacteria_u_p, Euryarchaeota, Acidobacteria, Saccharibacteria, Verrucomicrobia, Proteobacteria, Lentisphaerae, Spirochaetae, Peregrinibacteria, Cyanobacteria, Patescibacteria, Ws2, Elusimicrobia, Chloroflexi, Deferribacteres, Hydrogenedentes, Deinococcus-thermus, Tm6 (dependentiae), and Microgenomates. Relative abundance of all phyla was <0.01 in most of samples.

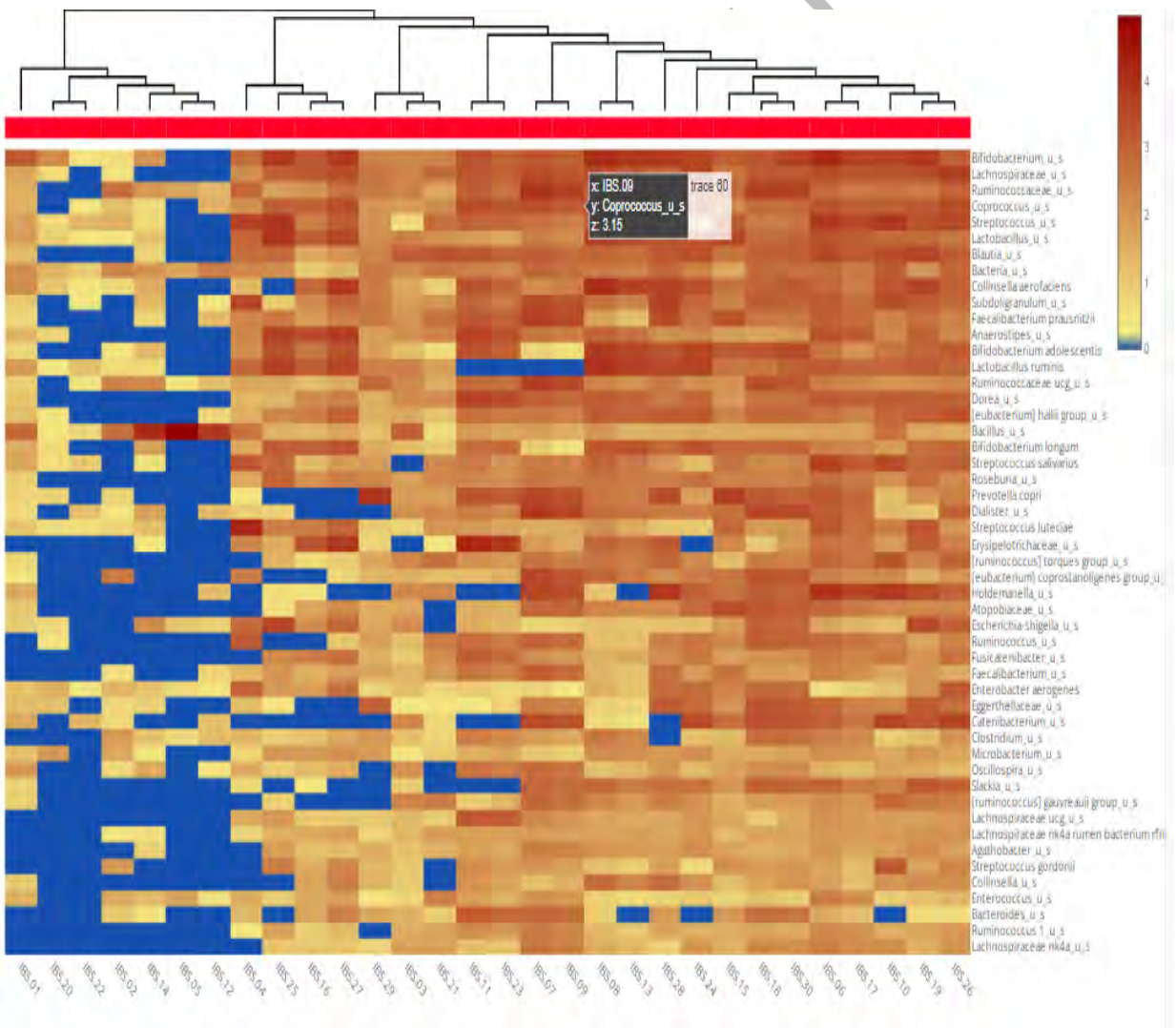


Figure 4.8: Relative abundance of bacterial phyla in IBS patients

4.4.5.4 High relative abundant phyla in IBS patients

Five important phyla that are present in all samples were Firmicutes, Acinetobacter, Proteobacteria, Bacteroidetes, Tenericutes and some unpredictable bacteria**.**

Firmicutes are gram positive bacteria that are normally present in gut but increase ratio of firmicutes have a bad effect over human health, Firmicutes ratio was higher in all patients mostly except IBS 2, IBS 12 and IBS 20. Highest ratio of Firmicutes was present in IBS 1(94.945%), IBS 3(97.23%), IBS 5(99.45%), IBS 14(97.0%), IBS 21(86%).

Second important genera present was **Actinobacteria.** These have low G-C content gram positive rods, they are divided into two classes depending upon mode of respiration as anaerobic bacteria, that include three main anaerobe families (Bifidobacteria, Propionibacteria and Corynebacteria) and an aerobe family (Streptomyces). The most represented in the human gut are Bifidobacteria. As they normally inhabit intestinal track of humans, they are nearly present in all IBS patients but most prevalent in IBS 8(48.87%) and IBS 13(46.44).

Next abundant phyla present in IBS patient was **Bacteroidetes**, which are gram-negative bacteria usually associated with production of short chain fatty acids (SCFAs) in gut as they can ferment polysaccharides and otherwise indigestible carbohydrates, in this way playing a role in maintaining healthy microbiota, overall very low amount of bacteriodetes were present in IBS patents yet some patients do have this phyla in low abundance i.e. IBS 7(14.72%), IBS 9(16.10%), IBS 15(21.21%), IBS 18(6.77%) and IBS 29(16.52).

Proteobacteria are gram negative pathogenic bacteria that are normally associated with intestinal gut mobility IBS 2 (39.42%), IBS 12(88.5%), IBS 20(59.63%), and IBS 22(67.93%) have high number of Proteobacteria which might suggest that these patient may have high chance of having any gastric infection problem.

Another phyla present in one of IBS patient was **Tenericutes,** this phylum contain gram–negative, obligate cell-associated bacteria that have lost their cell wall and are sometime present in gut , IBS 17(4.53%) have some abundance of this phylum.

Unpredictable or novel bacterial phyla were also reported in some patients IBS 22 have 5.37% unclassified bacterium phyla , similarly IBS 21, IBS 26 IBS 28 IBS 10 IBS

17 IBS 18 also have number of these phyla, which need to be studied further in order to report their role in IBS progression.

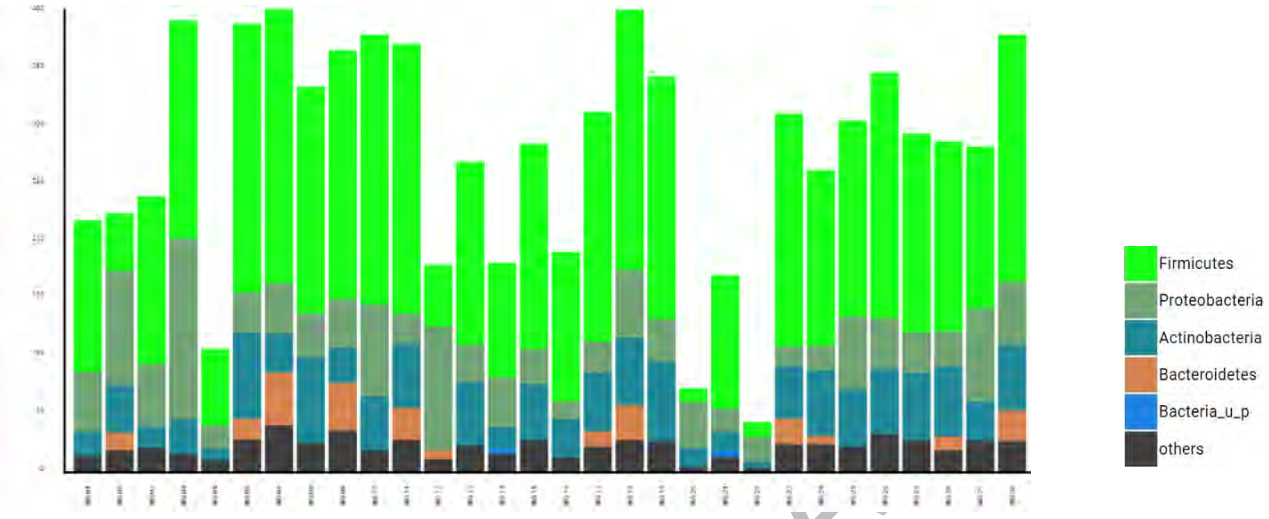


Figure 4.9: High relative abundant phyla in IBS patients

4.4.5.5 Genus level diversity in IBS patients

Bacillus, Bifidobacterium, Brevibacillus, Catenibacterium, Lactobacillus, Prevotella, Sphingomonas, Streptococcus, Anaerostipes, , Anoxybacillus, Bacillus, Bifidobacterium, Blautia, Brevibacillus, Catenibacterium, Collinsella, Coprococcus, Dialister, Eggerthellaceae, Erysipelotrichaceae, Faecalibacterium, Holdemanella, Lachnospiraceae, Lactobacillus, , Lysinibacillus, Microbacterium, Nitrobacteria, Peptostreptococcaceae, Prevotella, Rhodobacteraceae, Ruminococcaceae, Sphingomonas, Streptococcus, SubdoligranulumU114, and many others which are in lower concentration. We will discuss only those species which are in greater abundance because they have effect on gut microbial diversity.

4.4.5.4 Strain level diversity in IBS Patients

Anaerostipes_u_s, Anoxybacillus kestanbolensis, Aurantimonas altamirensis, Bacillus_u_s, Bacillus cereus, Bacillus cereus atcc 10987, Bacteria_u_s, Bifidobacterium_u_s, Bifidobacterium adolescentis, Bifidobacterium longum, Blautia_u_s, Brevibacillus invocatus, Catenibacterium_u_s, Collinsella aerofaciens, Coprococcus_u_s, Dialister_u_s, Dorea_u_s, Enterobacter aerogenes, Erysipelotrichaceae_u_s, Faecalibacterium prausnitzii, , Holdemanella_u_s,

Lachnospiraceae_u_s, Lactobacillus_u_s, Lactobacillus ruminis, Nitrobacteria hamadaniensis, Ochrobactrum_u_s, Peptostreptococcaceae_u_s, Prevotella copri, Rhodobacteraceae_u_s, Rhodococcus_u_s, Ruminococcaceae_u_s, Ruminococcaceae ucg_u_s, Sphingomonas_u_s, Streptococcus_u_s, Streptococcus luteciae, Streptococcus salivarius, Subdoligranulum_u_s, U114_u_s[eubacterium] hallii group_u_s

4.4.5.5 IBS sub types and microbial diversity

IBS is sub divided into three types, IBS C IBS D and IBS M depending upon symptoms, three types IBS has difference in gut microbial diversity.

Table 4.3 Kruskal-Wallis (pairwise) analysis showing p, H and q values between IBS subtypes.

Group 1	Group 2		H	p-value	q-value
Constipation (n=6)	Diarrhea (n=9)		1.388889	0.238593	0.483449
Constipation (n=6)	Mix (n=14)		0.979592	0.3223	0.483449
Diarrhea (n=9)	Mix (n=14)		0.120099	0.728927	0.728927

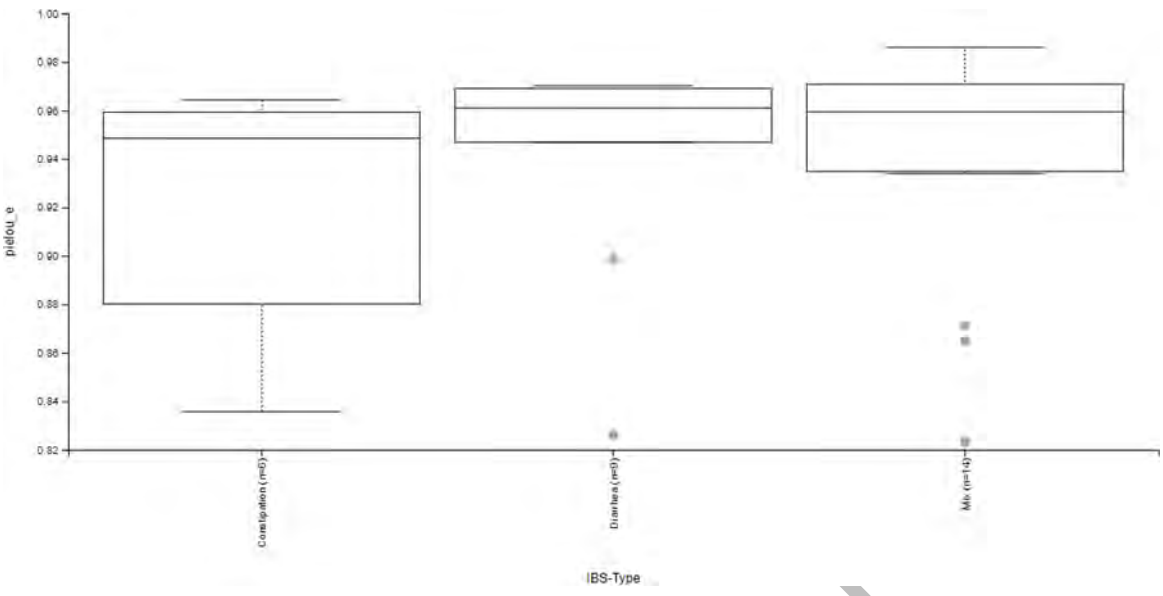


Figure 4.10 :Alpha diversity box plot- Kruskal-Wallis (pairwise) analysis showing evenness group significance.

4.4.5.6 Phylum level diversity

At phylum level different subtypes of IBS have some difference in diversity of microbiota at phylum level, (**H 0.511220580186091, p-value 0.774443718380459). IBS M** have more common phylum like Firmicutes, Proteobacteria, Cynobacteria, Epsilonbacteraeota, Firmicutes, Actinobacteria, Bacteroidetes, Tenericutes, Bacteria_u_p, Lentisphaerae and verrucomicrobiota. Firmicutes were present in highest number in all patients suffering from IBS-M, while second most prevalent phylum was Actinobacteria.

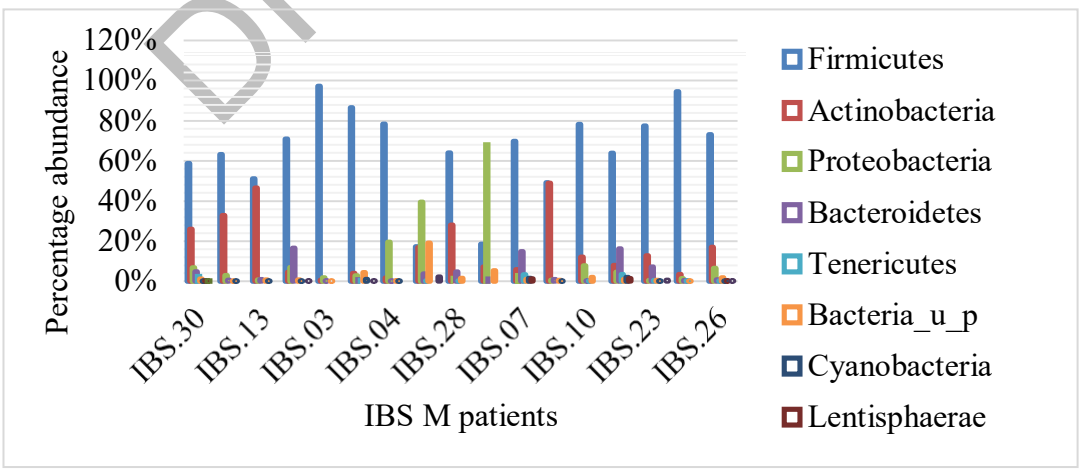


Figure 4.11: Graph showing phylum level diversity in IBS patients group IBS M.

IBS-C have Proteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, Fusobacteria, Bacteria_u_p and Firmicutes. Actinobacteria and firmicutes were present in higher concentration. In IBS-D diversity was least most abundant phyla present were Ignavibacteriae, Brc1, Planctomycetes, Tenericutes, Actinobacteria, Proteobacteria, among these proteobacteria and fermicutes were more common.

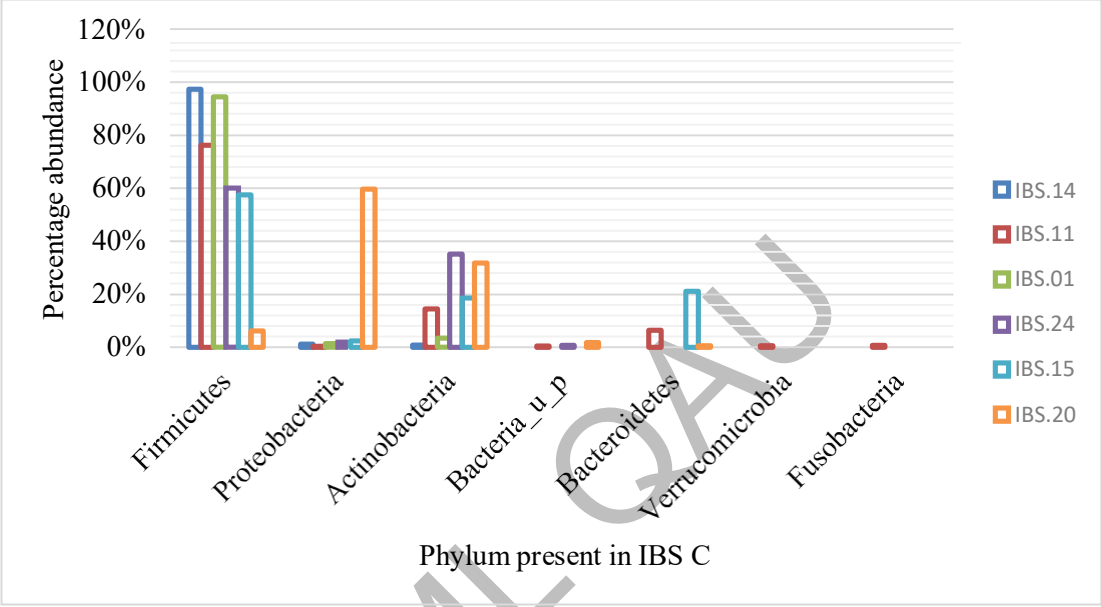


Figure 4.12 : Graph showing phylum level diversity in IBS patients group IBS C.

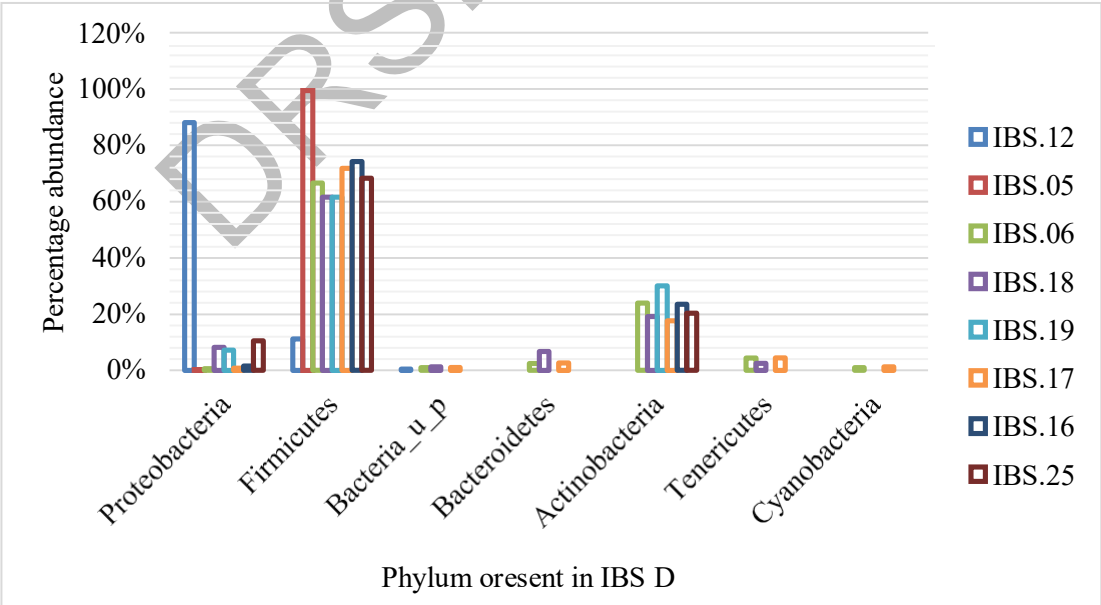


Figure 4.13 : Graph showing phylum level diversity in IBS patients group IBS D.

Overall diversity is same in all IBS subtypes, some variations were observed between Constipation and Diarrhea ($p < 0.238593$) , constipation and mixed type ($P< 0.3223$) and constipation and mixed type IBS $p< 0.728927$.

In general, this data suggest that there was relitive high abundance of proinflammatory bacterial species like Enterobacteriaceae, while reduced number of lactobacillus and bifidobacterium was observed. Moreover Bifidobacterium genus, Clostridiales order, Ruminococcaceae and Erysipelotrichaceae families, all short chain fatty acids (SCFAs) producers were also observed to be lower in IBS patients.

Some of the species of phylum firmicutes like streptococcus spp was high in patients having high severity score. Which can be linked to increased level of dysbiosis in most of IBS patients, these increased level of streptococcus species also suggest increase level of IL-6, which shows inflammation in these patients. Some probiotic strains of phylum Actinobacteria were also predominant in IBS patients like Bifidobacterium and Collinsella. As compared to IBS D and IBS M, constipation predominant patients have lower level of IBS.

Archea containing methanogens, which help to convert hydrogen produced in gut to methane was also seen in some IBS patients, mostly which were constipation related IBS cases.S o IBS-C have an increase level methanogens. Methanobrevibacter, was present in IBS-C subtype. Some of important bacterial taxon summary present in IBS patients were:

Table 4.4 Overveiw of bacterial species in IBS patients.

Taxa	Relative abundance
Enterobacteriaceae	Higher
Lactobacillus genus	Higher
Bifidobacterium	Lower
Firmicutes/Bacteroides	Higher
Clostridiales	Higher
Ruminococcaceae or Ruminococcus	Higher
Erysipelotrichaceae	Lower
Methanogens	Lower
Veillonella	Higher
Faecalibacterium	Lower
Lachnospiraceae	Higher

4.4.5.7. Gut microbiota and the link to IBS symptoms

Most of IBS patients were having symptoms of deregulation, pain, constipation, bdominal distention, blotting, some were also having psychaiatric problems, level of stress was high in IBS patients. Its difficult to predict gut microbial relationship to level of symptoms severity, but a positive corelation was observed in patients having high stress level and presence of benefical bacterial species, e.g in patients having high stress level bifidobacterium concentration was decreased. Another important finding was abdominal pain related to presence of desrease level of Firmicutes phylum. Patients having abdominal pain was having low level of firmicutes.

Vitamin D level was low in 82% of IBS subjects,(Kang *et al*., 2014) ANCOM volacano plot showed bacterial phylum diversity that have high correlation with with low level of vitamin D level, some species related to low level of vitamin D belongs to phylumtenericutes, with confidence interval 0.999984143. ANCOM statistical results shows common feature ID related to low level of vitamin D weref66b30724831008e3dc3f25353396999 and 4ef43bbda62ec6289c25f72c8f735c0f 4 having W value 522 and 410 respectively.

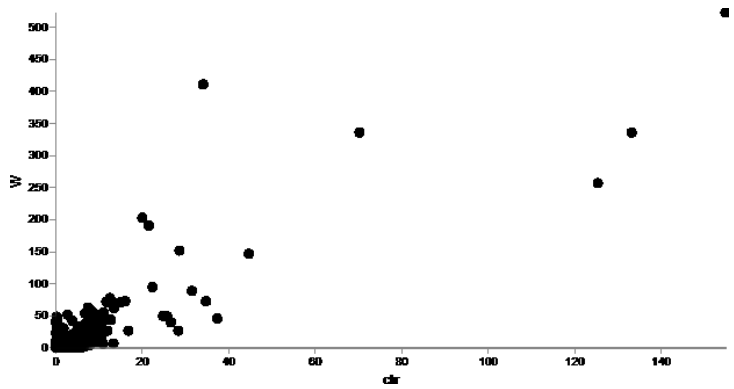


Figure 4.14: ANCOM Volcano Plot showing relationship of low level of vitamin D level with bacterial phylum present.

Depression changes gut microbial diversity among IBS patients, interesting interactions were found between many bacterial species and patients having high depression level, most prevalent phylum present was Enterobacteriaceae(Jiang *et al*., 2015). Most of firmicutes phylum was related to high level of depression in IBS patients, genus of firmicutes that were present in abundance having depression in patients: Weissella, bromii, belonging to family Leuconostocaceae and Ruminococcaceae (Hu *et al*., 2019). Other phylum related to IBS was proteobacteria family Aeromonadaceae genus Aeromonas.

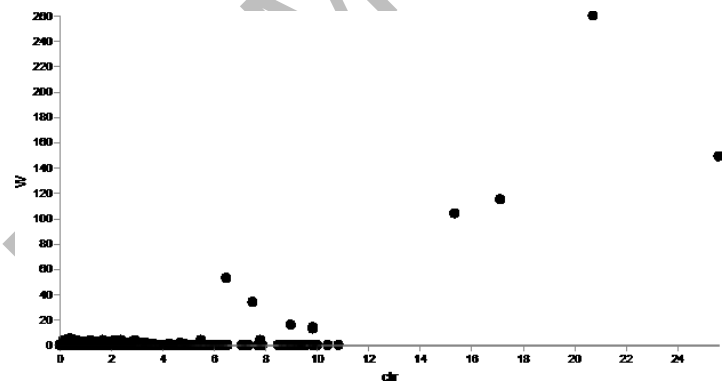


Figure 4.15: ANCOM volcano plot ANCOM volacano plot showed bacterial phylum diversity that have high correlation with varying level depression in IBS patients.

Discussion

Culturing based techniques only give a narrow picture of gut microbiota, out of 50 bacterial phyla only 29 are possible to culture(Hongoh, Ohkuma, & Kudo, 2003). From these phyla ten are more predominate in human gut, most of species living in gut belongs to phyla Firmicutes and Bacteroidetes (Dethlefsen, McFall-Ngai *et al*. 2007. With

advance in technology now 16 S gene sequencing studies are become more predominate while analyzing gut microbiota of IBS patients (Watson *et al*., 2019).

Our study revealed presence of five phyla that were more predominate in IBS patients, out of which most species are of Firmicutes and Bacteroidetes. Results from previous studies have somehow varying results but all have a common conclusion like microbial signatures of IBS patients changes as that of normal. Dysbiotic microbiota can be characterized at phylum level.

Different studies have different results one from (Jalanka-Tuovinen *et al*., 2014; Joo, 2015). (Rajilić-Stojanović *et al*., 2015) reports to have higher level of proteobacteria in IBS patients. Study showed higher level of Veillonella Lactobacillus and Ruminococcus in IBS (Collins, 2016) while that of Bifidobacterium, Faecalibacterium, and methanogens were reduced in IBS patients that is similar to our study. Three studies from past showed presence of higher level of Firmicutes/Bacteroidetes in patients(Manichanh *et al*., 2014) which is also in accordance to what we found. Studies from past have some varying results (Faith *et al*., 2013) because its difficult to predict gutmicrobiota accurately. Similarly lower and higher percentages of Eubacterium rectal were found in two studies(G. Parkes *et al*., 2012).

Another important order found in IBS patient was clostridia which is in consistence with a report (Maukonen *et al*., 2006). So, we can not say with 100% confident which phyla will show alteration in IBS patients, due to data history of IBS available online(Labus *et al*., 2017). Although difference are there in fecal microbial composition of IBS patients(Xu *et al*., 2019), but on general some studies showed relative consistent tendencies for variation while other shows differences in results(Y. Liu *et al*., 2016).

Scientist now come up with five major phyla that make gut composition in IBS patients which includes Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Tenericutes, and Fusobacteria(Hollister *et al*., 2020), this is in accordance with our results. We also found Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Tenericutes, Fusobacteria and some number of unpredictable bacteria in all IBS samples.

Out of all these phyla most of species present in IBS patients belong to Firmicutes and Bacteroidetes, previous studies also proven this fact that main colonizing bacteria of human gut belongs to these phyla(Maharshak *et al*., 2018). In our study Firmicutes level

was high in IBS M, IBS D and IBS C, while Bacteroidetes were high in IBS D and IBS C. From literature we found some how same studies firmicutes to Bacteroidetes ratio was 1.2-3.5 fold higher in IBS patients(Giamarellos-Bourboulis *et al*., 2015).

Within phyla level when we go down uptill class level previous studies show higher level of clostridia species(Nagel, Traub, Allcock, Kwan, & Bielefeldt-Ohmann, 2016), Scientist believe that these changes in phylum level especially firmicutes phyla are consistent through downstream taxonomical hierarchy(Zeber-Lubecka *et al*., 2016), similar trend was observed in this study. In phylum firmicutes when we go further down to specie level most of them belongs to family Ruminococcaceae (Xu *et al*., 2019), this family is predominant in present study also. another important class present in our data was bacillia which also belong to phylum firmicutes , in this most abundant was Lactobacillaceac at family level and lactobacillus at genus level, study conducted by (Leite *et al*., 2020) also show same results.

As mentioned above, the second most abundant phylum in the human gut, Culturing based techniques only give a narrow picture of gut microbiota, out of 50 bacterial phyla only 29 are possible to culture(Hongoh *et al*., 2003). From these phyla ten are more predominate in human gut, most of species living in gut belongs to phyla Firmicutes and Bacteroidetes (Dethlefsen, McFall-Ngai *et al*. 2007. With advance in technology now 16 S gene sequencing studies are become more predominate while analyzing gut microbiota of IBS patients (Watson *et al*., 2019).

Our study revealed presence of five phyla that were more predominate in IBS patients, out of which most species are of Firmicutes and Bacteroidetes. Results from previous studies have somehow varying results but all have a common conclusion like microbial signatures of IBS patients changes as that of normal. Dysbiotic microbiota can be characterized at phylum level. Different studies have different results one from (Jalanka-Tuovinen *et al*., 2014; Joo, 2015). (Rajilić-Stojanović *et al*., 2015) reports to have higher level of proteobacteria in IBS patients. Study showed higher level of Veillonella Lactobacillus and Ruminococcus in IBS (Collins, 2016) while that of Bifidobacterium, Faecalibacterium, and methanogens were reduced in IBS patients that is similar to our study.

Three studies from past showed presence of higher level of Firmicutes/Bacteroidetes in patients(Manichanh *et al*., 2014) which is also in accordance to what we found. Studies

from past have some varying results (Faith *et al*., 2013) because its difficult to predict gutmicrobiota accurately. Similarly lower and higher percentages of Eubacterium rectal were found in two studies(G. Parkes *et al*., 2012). Another important order found in IBS patient was clostridia which is in consistence with a report (Maukonen *et al*., 2006). So, we can not say with 100% confident which phyla will show alteration in IBS patients, due to data history of IBS available online(Labus *et al*., 2017).

Although difference are there in fecal microbial composition of IBS patients(Xu *et al*., 2019), but on general some studies showed relative consistent tendencies for variation while other shows differences in results(Y. Liu *et al*., 2016). Scientist now come up with five major phyla that make gut composition in IBS patients which includes Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Tenericutes, and Fusobacteria(Hollister *et al*., 2020), this is in accordance with our results. We also found Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Tenericutes, Fusobacteria and some number of unpredictable bacteria in all IBS samples.

Out of all these phyla most of species present in IBS patients belong to Firmicutes and Bacteroidetes, previous studies also proven this fact that main colonizing bacteria of human gut belongs to these phyla(Maharshak *et al*., 2018). In our study Firmicutes level was high in IBS M, IBS D and IBS C, while Bacteroidetes were high in IBS D and IBS C. From literature we found some how same studies firmicutes to Bacteroidetes ratio was 1.2-3.5 fold higher in IBS patients(Giamarellos-Bourboulis *et al*., 2015).Within phyla level when we go down uptill class level previous studies show higher level of clostridia species(Nagel *et al*., 2016), Scientist believe that these changes in phylum level especially firmicutes phyla are consistent through downstream taxonomical hierarchy(Zeber-Lubecka *et al*., 2016), similar trend was observed in this study.

In phylum firmicutes when we go further down to specie level most of them belongs to family Ruminococcaceae (Xu *et al*., 2019), this family is predominant in present study also.another important class present in our data was bacillia which also belong to phylum firmicutes , in this most abundant was Lactobacillaceac at family level and lactobacillus at genus level(Leite *et al*., 2020) but in our study lactobacillus was lower in abundance which is in accordance with (Carroll *et al*., 2010). Most of the species in Lactobacillus and Bifidobacterium genera made relationship with other gut microbiota by which they play a role in gut modulation and immune system, moreover they also secrete certain

compounds (bacteriocins) which have ability to destroy pathogenic bacteria, mostly against Salmonella and Listeria (Angelakis, Merhej, & Raoult, 2013). They also made interactions with immune cells CD209, with help of dendritic cells thus helping in immune system to work efficiently(Pace, Pace, & Quartarone, 2015).

Second prevalent phyla was gram negatives Bacteroidetes, among this class Bacteroidia, genus Prevotella proportion was high. Arumugam *et al*. and Liu *et al* consider these as one of three enterotype in human gut (B. Wang *et al*., 2020), (Carroll, Ringel- Kulka, Siddle, & Ringel, 2012). Bacteroidetes having variable diversity in gut leading from lower (Noor *et al*., 2010) to highest among different IBS population.(Krogius-Kurikka *et al*., 2009). Some of Previous studies reported majority of IBS patients have altered gut composition on basis of firmicutes and Bacteroidetes ratio(G. Parkes *et al*., 2012), when firmicutes level increases Bacteroidetes decreases(Maukonen *et al*., 2006). Most of species belonging to Bacteroidetes are either beneficial or non-beneficial characteristics (Wexler, 2007) so depending upon prevalence of beneficial or non-beneficial microbiota, this phylum have a very important role in IBS progression. As number of non-beneficial bacteria increases symptoms severity in IB patients increases mostly related to pain sensation(Bai *et al*., 2019).

Gram positive bacteria Actinobacteria includes mostly species related to probiotic productions , this genera is reported to be less prevalent in our study, previous studies on prevalence of probiotics related genera in IBS also shows lower abundance(Z. Wang *et al*., 2019).

Among IBS subtype actinobacteria have lower prevalence in IBS-D subtype, another study also supports this result (Krogius-Kurikka *et al*., 2009).Bifidobacterium present in this phylum have a very positive impact on gut mucosal barrier, so decrease in level of this bacteria in IBS can be related to leaky gut phenomena(Zapater, Such, & Sanz, 2013). Overall inconsistency suggests that these probiotic species beneficial traits might have a role in gut IBS patients, more studies are needed to clear impact(Bron *et al*., 2017).

Several studies found association of proteobacteria in IBS patients gut(Carroll *et al*., 2012). We found proteobacteria as third predominant genera in IBS patients, among this most important order reported was Enterobacteriales and family Enterobacteriaceae.(Chung *et al*., 2016) also comes up with same finding in there IBS gut microbiome project.

Although *Firmicutes*/*Bacteroidetes* can be a rough indicator of bacterial dysbiosis in gut but both high(Rajilić-Stojanović, Dimitrijević, & Golić, 2019) and low(Tana *et al*., 2010) ratio have been described in IBS patients. Scientist proposed different hypothesis to explain these in consistency in results, leading from technical issues (sequencing type/ DNA extraction methods)(Rigsbee *et al*., 2012) to experimental designs(choosing sample size and patients symptoms severity)(Menees & Chey, 2018).Due to these drawbacks in studies microbiota data manipulation is limited to phylum level study. We can improve genus-specie level study by use of new metagenomic bioinformatics analysis which will help in getting true picture of disease gut dysbiosis.

Short chain fatty acids are end product of microbial metabolism, Short-chain fatty acid like butyrate and acetate have benefical role in modulation of cell proliferation, giving energy, reducing gut inflammtaion as well as in protection against GI infections. Many bacterial species in gut are related to production of SCFAs e.g, clostridium, Eubacterium hallii and Eubacterium desmolans, these SCFAs producing bacteria inhibit growth of certain pathogenic bacteria *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and *E. coli*, (Duncan, Hold, Barcenilla, Stewart, & Flint, 2002).

One of the important functions performed by SCFAs producing microorganism is lowering pH in colon mainly this property is related to firmicutes, due to lower level of Ph bacterial species growth is also got effected being lower number of SCFAs producing microorganism (Barcenilla *et al*., 2000). Same was case in our study firmicutes ratio was high which might lead to conclusion that SCFAs producing bacteria level reduces in IBS patients. Some of the studies proved varying amount of clostridium in IBS patients.

One of other important bacterium associated with production of acetate and lactate is bifidobacterium adolescentis, presence of this bacterium indicates increase level of SCFAs in gut(Hatayama, Iwashita, Kuwajima, & Abe, 2007), in our study we didn't found this type of bacterium which is another indication of lower SCFAs production in IBS patients. Contradiction to this many studies also prove no significance difference in SCFAs producing bacteria and IBS patients (Peng, He, Chen, Holzman, & Lin, 2007).

As mainly clostridia class is associated with SCFAs production in IBS but varying results are observed showing both increase or decrease in their presence. *Ruminococcus* spp is mostly observed to be increased in patients with IBS same is the case with our study (Moore, CATO, & HOLDEMAN, 1972).One of the study also prove

lower level of bacteriodetes spp at lowe PH value (Ohkusa, 2019), this also proves our finding we also observe lower level of bacteriodetes in many IBS patients.

Constipated related diseases mainly IBS has association with methane production, in a healthy individual 30-40% methane production is observed (Bjørneklett & Jenssen, 1982). Another important finding is association of IBS and methane producing bacteria. This methane producing property is limited to methanogens which are involved in conversion of hydrogen to methane, these bacteria belong to kingdom Archea (Lepp *et al*., 2004). Methano bacteriales is most abundant methane producer in human gut which helps not only in anti-inflammation but also in help in transfer of molecules across intestine, one of the study give an interesting finding is methane production in constipated patients and microbial richness (Kalantar-Zadeh, Berean, Burgell, Muir, & Gibson, 2019), according to this study presence of methanogens and enterotypes like *Clostridiales* or *Prevotella* species increase symptoms severity of IBS patients. These two species were also present in abundant in our study claiming presence of methanogens in our patients. Despite the robust connotation with clinical consequence, this gut microbiota cannot yet be described by genetic factors, differences in diet or the use of medications. And in IBS patients there is necessity for high quality clinical trials to examine methane as a biomarker for the diagnosis.

Vitamin D plays an important role in innate immune system, it not only changes transcription of cathelicidin but also deactivate beta 4 defensin mechanism, most of cell types in innate immune system have receptors for vitamin D level (Nwosu, Maranda, & Candela, 2017a). Vitamin D deficiency is mostly related to dysbiosis of gut microbial community many reports found its association with severe colitis (Sikaroudi *et al*., 2020). One of the studiesreported lower level of vitamin D in most of IBS patients with a significant difference of p=0.025, vitamin D have both direct and indirect effect in modulation of gut microbiome by releasing bacteriocins. Its also helps in reducing inflammation (Barbalho, Goulart, Araújo, Guiguer, & Bechara, 2019). These results can have a therapeutic role in controlling of IBS, vitamin D supplementation helps in reduction of IBS symptoms (El Amrousy, Hassan, El Ashry, Yousef, & Hodeib, 2018).

Chapter 5: Study of pathogenesis of culturable microbiota from IBS patients by using Pangenome analysis.

5.1. Introduction

Culturing is gold standard and traditional method of studying microorganism in laboratory, but only 2% bacteria can be cultured in laboratory (Wade, 2002). Genomic analysis is new technology which helps to better understand genetic makeup of unculturable bacteria, one can also study multiple micro-organisms from a single sample know as metagenomic analysis. Because of limitation of culturable methods pathophysiology of IBS is poorly understood , scientist are now using metagenomic analysis to understand role of microorganism in these patients (Spiller, 2007), by doing molecular analysis like PCR and targeted sequencing (G. C. Parkes, Brostoff, Whelan, & Sanderson, 2008). These short reads technologies also have some drawbacks, like it can lead to fragmented genome which is not suitable technique if we want to analyzes genome of a bacterial completely (Sasani, Cone, Quinlan, & Elde, 2018).

To overcome this drawback, from past few years long reads sequencing like (PacBio) and Oxford Nanopore Technologies (ONT) are also gaining attention as they give more accurate overlook of gut analysis (Liang *et al*., 2020). They produce long reads of tens kilobase pairs which is easy, speedy and cost-effective (Ameur, Kloosterman, & Hestand, 2019). MinION sequencer, is a portable device which is now commonly used for rapid and onsite analysis of bacterial genome (Castro-Wallace *et al*., 2017).

Despite of high prevalence of IBS worldwide, its pathophysiology is still unclear, previous studies usually focused on gastrointestinal dysbiosis in these patients, proving role of gut microbiota in progression of IBS but no study is there which shows the whole genome of bacterial isolates in IBS patients. By analyzing whole genome of some more prevalent bacterial organism from IBS we come up with functional pathways, virulence genes and antibiotic resistance gene of these isolates. This will help in more understandings of gut microorganism role in IBS patients.

As most of studies related to gut microbiota were carried out using 16S ribosomal RNA marker gene sequencing, our study will focus on functional metagenomics related to single species and strains that are more predominate in most of patients

which help in prediction of functional pathways changes in dysbiosis. As this study involve use of three techniques for identification of isolates, it gives comparison among Sanger sequencing, traditional culture-based study and long read analysis of isolates.

Culturable bacteria isolated from our IBS samples belong to both gram positive as well as gram negative bacteria. Among gram positive *Enterococcus* a large genus of lactic acid bacteria of the phylum Firmicutes was isolated. Enterococci are ubiquitous microorganisms having diverse ecological niches but mostly prominently in gastrointestinal tract of humans and animals (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). Production of enterocins make them used as probiotics, but in last few years their role as probiotic become ambiguous. This ambiguity in their probiotic role is related to presence of virulence factors and antibiotic resistance genes (Haney, Mansour, & Hancock, 2017).

Moreover, these virulence traits are also known to be transferred genetically which make them opportunistic pathogens in gastrointestinal track. These reports suggest serious concerns related to Enterococcus before using them as probiotics(Abd El-Baky, Ibrahim, Mohamed, Ahmed, & Hashem, 2020). In the present study Whole-Genome Sequencing (WGS) of Enterococcus spp was done for checking presence of resistanceand virulence genes, isolated from human gut.

Gram negatives mostly belongs to phylum proteobacteria including *Escherichia coli (E. coli),* Salmonella, Shigella, and other Enterobacteriaceae, Pseudomonas. From all these we pick up most predominant strains having role in gastrointestinal dysfunction, i.e *E.coli*, *Klebsiella pneumoniae, Shigella flexneri* and *Proteus mirabilis(G. C. Parkes et al., 2008).* Although these culturable are mainly normal gut flora which help body to resist against pathogenic bacteria, any alteration to these leads to inflammation and impair gastrointestinal tract. IBS is related to more severe gut inflammation, is known to be associated with dysbiosis that includes less number of Gram-positive and more of Gram-negative bacteria (Loh & Blaut, 2012). In addition, segmented filamentous bacteria (SFB) were also found to be higher in chronic inflammatory intestinal diseases (Loh & Blaut, 2012).

In this phase of our study single bacterial genome using oxford nano pore technology MinION, we identified complete genomes of some bacterial isolates from fecal

samples of IBS patients which were used to identify presence of virulence factors and antibiotics resistance genes in these isolates and functional metagenomics of single species that were more predominant in IBS patients.

5.2. Aim and Objectives

This study aim was to check different type of culturable bacteria present in gut of IBS patients, using gold standard culturing method, objectives of study were:

- To isolate and characterized culturable bacteria from IBS patients.
- To evaluate the genetic attributes of selected represented strains.

5.3. Material and Methods

5.3.1. Preparation of stool samples

Stool samples were stored at -20°C , right after collection, microbiological analysis of stool samples was done. From stool samples first aim was to isolate culturable bacteria, steps were followed:

1. Freeze samples were placed in hot water bath for some time to bring them to normal temperature.

2. Loop full of these samples were added in TTB (tetrathionate) glass bottle.

3. Placed these in incubator for 24 hours.

4. Next day these were cultured on blood and MacConkey agar.

5.3.2. MacConkey Ager Preparation

MacConkey agar (MAC) was established by Alfred Theodore MacConkey in 20th century. This is first differential and selective solid media used for isolation of gram-negative rods, especially members belonging to class Enterobacteriaceae and the genus Pseudomonas. Media was prepared by following steps:

1. MacConkey agar was prepared by adding 49.53 g into 1000 ml of distilling water followed by autoclave. Sterilization conditions were heating up to 120oC for 15 mins.
2. Media was poured into the Petri plates. Plates were incubated for 16 hrs at 37oC to avoid contamination.
3. After 16 hrs. media was inoculated with the sample.

5.3.3. Blood Agar preparation

Blood agar supports growth of fastidious bacteria and used to differentiate organism on basis of their hemolytic properties. Blood agar is highly nutritious medium having tryptic soy Agar or Columbia Agar having 5 % horse blood. Samples were streaked on MacConkey and blood agar, incubated for 16 hrs. at 37°C followed by several streaking cycles to isolate pure colonies.

5.3.4. Identification/Phenotypic characterization of Gram-Negative Bacteria

Colonies on MacConkey agar were identified using colony morphology and gram staining followed by a series of biochemical tests. Three different types of colonies were observed from different samples:

1. **Strong lactose fermenters**

Bacteria like Escherichia, Klebsiella, and Enterobacter tend to produce red color colonies and have a clear zone of bile precipitation around there colonies.

2. **Slow lactose fermenters**

Some colorless or light pink color colonies were observed in many samples, these can be of Citrobacter, Providencia, Serratia, and Hafnia.

3. **Non-lactose fermenters**

Species of Proteus, Klebsiella, Salmonella, and Shigella appear colorless and transparent on agar.

5.3.4.1. Calculating colony forming unit (CFU/g)

After defined time of incubation, all the plates were examined for microbial growth. Color, size, and morphology of the microbial colonies were noted. Total number of colonies on each plate were recorded by using colony counter. An average of 30 – 300 colonies were used for actual colony count. Microbial count of fecal samples was calculated by using CFU formula, colony forming unit/g of fecal material.

CFU/g = (number of colonies × dilution factor) ÷ volume plated in ml

5.3.4.2. Gram staining

Further confirmation was done using gram staining.

1. Colonies were heat fixed on the slide and Crystal violet stain was poured over the slide and wait for 1 min. Rinse slide by distilling water. Add iodine on the slide and again wait for 1 min then wash slide by distill water.

3. Now tilt the slide and pour decolorizer (ethanol) for 5 seconds.

4. Now add safranin on the slide and wait for 1 min and wash slide with distilled water, air dries the slide.

5. The slide was observed under a light microscope at X100 power. Different areas of the slide were examined. Gram negatives were stained pink.

5.3.5. Biochemical tests

Further after microscopy and colony morphology result were further confirmed by certain biochemical test. BioMérieux's API 10 S identification products kits were used for manual identification of gram-negative bacteria. Colonies were stored at -80 till DNA extraction.

5.3.6. DNA extraction and Gel electrophoresis

Favrogen mini stool DNA isolation kit was used to extract DNA from bacteria(As per manufacturer instructions).

5.3.6.1. Gel preparation

We use 1% gel, to visualize DNA. For 1% gel preparation:

1. 1 gram of agarose was added to 90 ml of distilled water, followed by 10 ml of TBE buffer. For mixing well, mixture was placed in microwave for 30 seconds.

2. For DNA visualization, 4 µl of ethidium bromide was added after cooling of gel.

3. Poured gel onto dish having comb, allow that to solidify, Dish was then transferred to tank having 1X TBE buffer.

4. Comb was detached, and 3µl of DNA mixed with 2µl bromophenol blue dye was loaded in comb holes. Gel was run for 35 mins at 120 volts and 400mA, followed by gel observation under UV.

5.3.7. PCR against 16S RNA gene primer

16S rRNA gene primer was designed to confirm our colony identification of bacterial pathogens. The QIAquick system uses a simple bind-wash-elute procedure (see flowchart "QIAquick and MinElute procedure"). Binding buffer is added directly to the PCR sample or other enzymatic reaction, and the mixture is applied to the QIAquick spin column. The binding buffer contains a pH indicator, allowing easy determination of the optimal pH for DNA binding (see figure "pH Indicator Dye"). Nucleic acids adsorb to the silica membrane in the high-salt conditions provided by the buffer. Impurities are washed away, and pure DNA is eluted with a small volume of low-salt buffer provided or water, ready to use in all subsequent applications.

5.3.8. Gel electrophoresis and purification

Gel was run to check size of PCR product using QIAquick Gel Extraction Kit (Cat. No. / ID: 28706X40, further gel purification was done using QIAquick PCR & Gel Cleanup Kit (Cat. No. / ID: 28506).

5.3.9. Preparing sample for sanger sequencing

Samples were sent to University of Minnesota genomic center UMGC for sanger sequencing**.** The UMGC provides Sanger sequencing on an Applied Biosystems 3730xl DNA Analyzer. This 96-capillary instrument affords rapid, high-throughput service with read lengths of up to 800-1000 bp.

5.3.10. Whole genome sequencing for selected strains

Whole-genome sequencing of selected strains were done using Oxford nanopore MinION technology using a protocol (Lambda-control-sqk-lsk109-CDE_9062_v109_revI). Representative strains were selected on basis of prevalence of these in all IBSsamples.

Sequence quality was assessed by FASTQC (Abd El-Baky *et al*., 2020),by online software https://epi2me.nanoporetech.com/workflow_instance.

5.3.11. Annotation and Antimicrobial resistance

FASTA files were uploaded at the RAST server for annotation, putative gene product identification (Abd El-Baky *et al*., 2020; Aziz *et al*., 2008). Moreover, to investigate the presence of antimicrobial resistance genes, the draft genome was uploaded at

Metagenomic Rapid Annotations using Subsystems Technology; genomes uploaded here can easily be accessed and analyzed by everyone.

To visualize and compare the genome with other published genomes at the time of analysis, the G-view server (https://server.gview.ca/) was also used (Abd El-Baky *et al*., 2020).

5.4. Results

5.4.1. Identification and physiochemical characterization of culturable microbes

Commonly present microbes in the human gut were cultured. Upon gram staining both positive and negative isolates were seen.

1.Gram positive bacteria

Cocci streptococcus and staphylococcus

a. Spore forming rods

Bacillus and Clostridium

b. Non spore forming rods

Corynebacterium , listeria, Actinomycesnocardia

2. Gram negative bacteria:

a. Cocci

Neisseria

b. Rods

Escherichia, Enterobacter, Serratia, Klebsiella, Salmonella, Shigella, Proteus Pseudomonas, Bacteroides

c. Curved

Campylobacter, Helicobacter, Vibrio

Table 5.1: Isolation of the Gram negatives / bile tolerant bacteria was done by using MacConkey /Blood agar.

Nu	Isolates	Nu	Isolates	Nu	Isolates
IBS 1	*1)Klebsiella pneumonia*	IBS 11	*1) Pseudomonas aeruginosa*	IBS 21	*1) Klebsiella pneumonia*
	2) Citrobacter koseri		*2) Shigellaa*		*2) E. coli*
IBS 2	*1) Proteus vulgarius*	IBS 12	*1) Pseudomonas aeruginosa*		*3) Enterococcus*
	2) Staphlococcus		*2) Staphlococcus*		*4) Proteus mirabilis*
IBS 3	*1) Shigella*	IBS 13	*1) Klebsiella pneumonia*	IBS 22	*1) E. coli*
	2) Citrobacter freundii		*2) Pseudomonas aeruginosa*		*2)staphlococcus*
	3) Proteus mirabilis		*3) Cinetobacter freundii*		*3) Enterococcus*
	4) Cinetobacter spp	IBS 14	*1) Shigella*	IBS 23	*1) E. coli*
	5) E. coli		*2) Pseudomonas aeruginosa*		*2) Enterococcus*
IBS 4	*1)Pseudomonas aeruginosa*		*3) E. coli*	IBS 24	*1)Pseudomonas aeroginosa*
	2) Shigella	IBS 15	*1) Shigella*		*2) Proteus mirabilis*
	3) Enterococcus		*2) Pseudomonas aeruginosa*	IBS 25	*1) Pseudomonas aeroginosa*
IBS 5	*1) Pseudomonas aeruginosa*		*3) Klebsiella pneumonia*	IBS 26	*1) Shigella*
	2) Proteus mirabilis	IBS 16	*1) Proteus mirabilis*		*2) Klebsiella pneumonia*
	3) E. coli		*2) Enterococcus*		*3) E. coli*
	4) Enterococcus	IBS 17	*1) Enterococcus*	IBS 27	*1) Proteus mirabilis*
			2)Klebsiella pneumonia		*2) Enterococcus*
IBS 6	*1) Proteus mirabilis*		*3) Proteus mirabilis*	IBS 28	*1) Pseudomonas aeroginosa*
	2) Citrobacter freundii	IBS 18	*1) Enterococcs*	IBS 29	*1) Pseudomonas aeroginosa*
	3) Shigella		*2) E. coli*		*2) E.coli*
	4) Enterococcus		*3) Proteus mirabilis*	IBS 30	*Staplococcus*
IBS 7	*1) Klebsiella pneumonia .*	IBS 19	*1) Klebsiella pneumonia .*		*2) Proteus mirabilis*
	2) Citrobacter freundii		*2) E. coli*		
	3) Staplococcus	IBS 20	*1) E. coli*		
	4) Enterococcus				
IBS 8	*1) Klebsiella pneumonia .*				
	2)Pseudomonas aeruginosa				
IBS 9	*1) Proteus mirabilis*				
	2) Staphlococcus				
IBS 10	*1) E. coli*				

5.4.2. Sanger sequencing results

On basis of most significant microbes present in gut 17 samples were selected for sanger sequencing, we send these isolates and get following results.

Table 5.2: Sanger sequencing results showing presence of different bacterial spp among IBS patients.

S.nu	Sanger Sequencing Results
1	Citrobacter/Klebsiella spp
2	Klebsiella spp
3	Citrobacter spp
4	*E. coli*
5	*E. coli*
6	*E. coli*
7	Klebsiella spp
8	Klebsiella spp
9	Klebsiella spp
10	Proteus spp
11	Enterococcus spp
12	Pseudomonas spp
13	Enterococcus spp
14	Enterococcus spp
15	Enterococcus spp
16	Enterococcus spp
17	Enterococcus spp

5.4.3. Whole genome sequencing

5.4.3.1. Gram positive isolates

We divide gram negatives in one group and gram positive in another and run MinION. Gram positive bacteria includes *Enterococcus. Enterococcus spp.* are commonly present in the human gut and are thought to be mostly safe and are used as probiotics. To characterize the isolated strains of *Enterococcus,* WGS was performed. Analysis of WGS sequencing data showed the presence of *E. faecalis, E. casseliflavus,* and *E. gallinarum*. Sequences were submitted to NCBI with Bio project number PRJNA682015. The enterococci were also tested for their antibiotic resistances and variant genes. Analysis of MinION data by EPITOME showed a difference in genome sizes of all strains suggesting that genetic variation is present within the *Enterococcus* strains.

5.4.3.1.1. Genome size and features

All four strains of *Enterococcus* showed different GC content ranging from 38% to 44%. The genome sizes also varied among strains (from 1,167, 642-3, 508, 906 bp). A complete summary of the genome report is attached as table 4 showing genome coverages, total read lengths in term of N50 and L50. Virulence and pathogenicity factors such as adhesins, invasions, pili, and hemolysin in *Enterococcus* make them potentially pathogenic for humans (Table 2). The circular map of *Enterococcus spp* was generated using G view server (figure 1) and a comparative genomic study was performed between different *Enterococcus* spp.

5.3: Genome details and GC content of various enterococcus strains (1 and 2).

Parameters	GC content	Genome size	N50	L50	Number of Contigs (with PEGs)	Number of Subsystems	Number of Coding Sequences	Number of RNAs
Enterococcus faecalis QAU 14	39.0	1,167,642	35578	13	38	157	3260	32
Enterococcus casseliflavus QAU 15	43.5	3,508,906	2296479	1	3	357	4867	75
Enterococcus gallinarum QAU 16	38.2	2,877,613	3451405	1	2	353	3754	77
Enterococcus gallinarum QAU 17	40.6	3,483,524	3451405	1	2	353	3754	77

Parameters	Total genes	CDSs (coding)	Genes Pseudo genes Total	Pseudo genes Frameshifted	Pseudo genes incomplete	Pseudo genes Internal stop	Pseudo genes Multiple problems
Enterococcus faecalis QAU 14	1,198	1,165	173	992	977	86	370	412
Enterococcus casseliflavus QAU 15	3,374	3,295	1,226	2,069	2,016	92	106	141
Enterococcus gallinarum QAU 16	2,843	2,753	1,270	1,483	1,398	87	78	79
Enterococcus gallinarum QAU 17	3,324	3,243	2,204	1,039	1,003	43	19	26

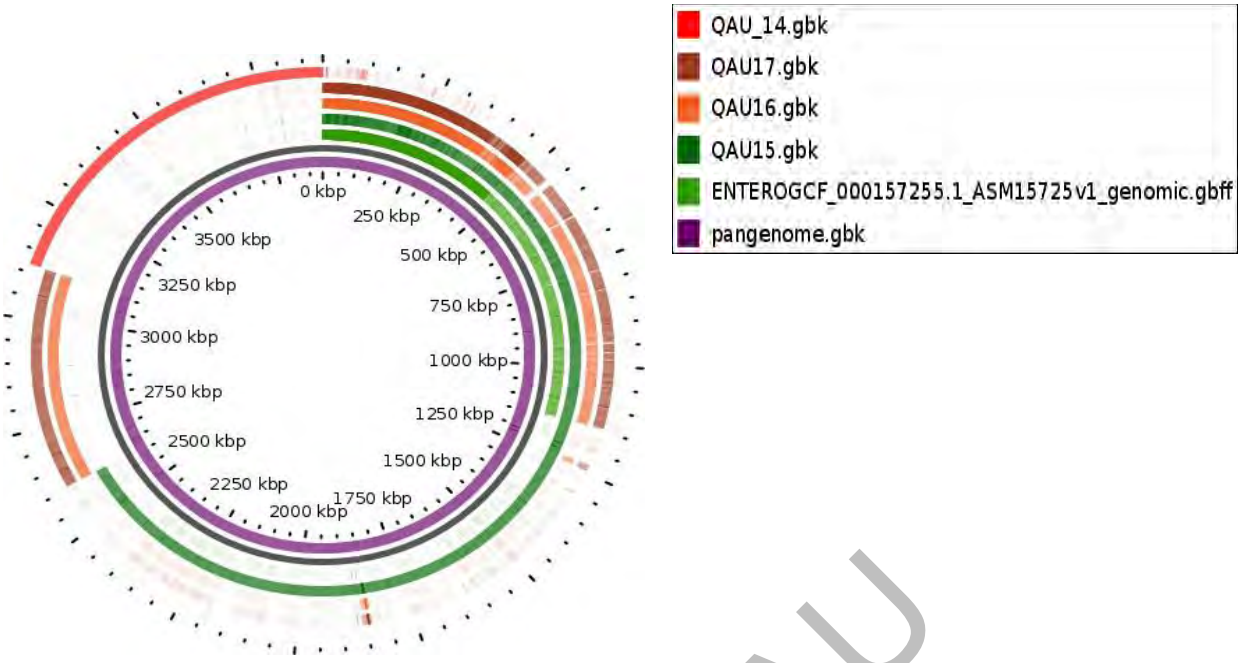


Figure 5.1: Circular map of *Enterococcus spp* showing comparative genomics between different species. QAU14 (*E. faecalis*), QAU15 (*E. casseliflavus*), QAU16 (*E. gallinarum*) and QAU17 (*E. gallinarum II*) with an already reported genome of Enterococcus on NCBI.

5.4.3.1.2. Metabolic network

The metabolic pathway/genome database (PGDB) was created computationally with KEGG metabolic pathways in RAST annotation server version 2020(Alcock *et al*., 2020). The genome size of all strains was between 1M-4M, showing size diversity among different strains. GC content also varied among strains ranging from 38%-44%. These sizes are in agreement with *Enterococcus spp* genome present on NCBI. We performed a different type of analysis on the genomic data to evaluate *Enterococcus* strain as a potential probiotic. Thirteen genes related to bacteriocin production were found in *E. casseliflavus* QAU15. Eight genes co-occur together in a cluster-based subsystem. One of these genes is responsible for producing colicin V (a bacteriocin produced by some strains of *Enterococcus*). Nine genes were present in two strains of *E. gallinarium* for bacteriocin production. Production of colicin V in these bacteria suggests their role in the progression of gastrointestinal infection. Many recent studies are now developing a relationship between pathogenicity and colicinogeny in different bacterial strains (Ozanne, Mathieu, & Baril, 1977).However,

the presence of bacteriocins only is not enough to declare *Enterococcus* as a non-suitable candidate for GRAS (generally regarded as safe). Hence, we did antibiotic resistance gene analysis of our strains; phenotypic data showed vancomycin resistance among a few strains of *Enterococcus*. Using RAST software, we found the presence of different antibiotic resistance genes but the most common was *vanXY*. This is of clinical significance as it is also expressed phenotypically in *E. faecalis* QAU14, *casseliflavus* QAU15, and *E. gallinarum* QAU16.

Table 5.4: Genetic characterization of *Enterococcus* strains on basis of WGS results shows the presence of different genes related to its subsystem features.

Subsystem features	*E. faecalis* QAU14	*E. casseliflavus* QAU15	*E. gallinarum* QAU16	*E. gallinarum* QAU17
Cofactors, vitamins, prosthetic groups, pigments	64	147	141	141
Cell wall and capsule	96	155	149	149
Adhesion	-	1	1	1
Toxins and superantigens	-	-	-	-
Bacteriocins, ribosomally synthesized antibacterial peptides	-	13	9	9
Resistance to antibiotics and toxic compounds	66	72	-	-
Virulence, disease and defense—no subcategory	-	-	-	-
Invasion and intracellular resistance	17	14	15	15
Potassium metabolism	-	11	10	10
Photosynthesis	-	-	-	-
Miscellaneous	4	34	19	19
Phages, prophages, transposable elements, plasmids	32	11	44	44
Membrane transport	57	99	84	84
Iron acquisition and metabolism	11	4	4	4
RNA Metabolism	57	137	120	120
Nucleosides and nucleotides	52	103	103	103
Protein metabolism	158	230	200	200
Cell division and cell cycle	7	54	42	42
Motility and chemotaxis	-	24	15	15
Regulation and cell signaling	24	44	41	41
Secondary metabolism	-	6	1	1
DNA metabolism	122	189	110	110
Fatty acids, lipids, and isoprenoids	4	99	75	75
Nitrogen metabolism	-	13	6	6
Dormancy and sporulation	-	4	2	2
Respiration	42	61	46	46
Stress response	40	77	60	60
Metabolism of aromatic compounds	-	2	2	2
Amino acids and derivatives	190	433	309	309
Sulfur metabolism	4	55	38	38
Phosphorus metabolism	23	32	33	33
Carbohydrates	331	859	657	657

5.4.3.1.3. Evaluation of Antibiotic Resistance

The comprehensive antibiotic resistance database was used to determine the presence of antibiotic resistance genes. *Enterococcus spp.* were then tested against commonly used antibiotics, e.g., Daptomycin, Gentamicin Vancomycin, Tigecycline, Streptomycin, Nitrofurantoin, Linezolid, and Ampicillin. Results show that *E. gallinarium* QAU17, *E. faecalis* QAU14, *E. casseliflavus* QAU15, and *E. gallinarum* QAU16 were resistant against vancomycin. *E. faecalis* and *E. gallinarium* were also resistant to linezolid while *E. casseliflavus* was resistant against ampicillin and vancomycin. We then used CARD software to find presence of antibiotic resistance genes so we can see the presence of antibiotic resistance genes in these isolates. *VanXYC* glycopeptide resistance gene cluster was observed in *E. gallinarium* suggesting a strong antimicrobial resistance pattern here.

Table 5.5: Antibiotics data, commonly used antibiotics tested against different enterococcus strains using Khyber disk diffusion method.

Antibiotics	***E. faecalis* QAU 14**	***E. casseliflavus* QAU 15**	***E. gallinarum* QAU 16**	***E. gallinarum* QAU 17**
Daptomycin	S	S	S	S
Gentamicin	S	S	S	S
Vancomycin	R	R	R	R
Tigecycline	S	S	S	S
Streptomycin	S	S	S	S
Nitrofurantoin	S	S	S	S
Linezolid	R	S	R	S
Ampicillin	S	R	S	S

E. gallinarium has *AAC*-6 (aminoglycoside acetyltransferase-Ii having role against aminoglycoside antibiotics) protein homology model belonging to the aminoglycoside class of antibiotics that work by antibiotics inactivation which is somehow expressed phenotypically that is its resistance towards vancomycin and linezolid. Three types of genes related to vancomycin resistance were found in *E. gallinarium* isolates i.e *vanRC*, *vanXYC* and *vanC,* which are protein homologs and act against glycopeptide antibiotics. These results were verified by *in vitro* analysis of drug susceptibility

testing; most strains were only resistance to vancomycin, suggesting vancomycin resistance as an intrinsic property of *Enterococcus* genome.

Table 5.6: Presence of different antimicrobial genes and class of drug resistance and their mechanism according to CARD based upon WGS data.

Factors	***E. gallinarium* QAU17**			***E. gallinrum* QAU16**	***E. casseliflavus* QAU15**
RGI criteria	Perfect	Strict	Strict	Strict	Strict
ARO term	*VanRC*	*VanXY*	*VanC*	ACC(6)-li	*VanXYC*
SNP					
Detection criteria	Protein homolog model	Protein homolog model	Protein homolog model	protein homolog model	protein homolog model
AMR gene family	glycopeptide resistance gene cluster, *vanR*	glycopeptide resistance gene cluster, *vanR*	glycopeptide resistance gene cluster, *vanR*	AAC(6')	glycopeptide resistance gene cluster, *vanXY*
Drug class	glycopeptide antibiotic	glycopeptide antibiotic	glycopeptide antibiotic	aminoglycoside antibiotic	glycopeptide antibiotic
Resistance mechanism	Antibiotic target alteration	antibiotic target alteration	antibiotic target alteration	antibiotic inactivation	antibiotic targ*et al*teration
% identity	100	99.4	98.83	99.4	79.8
% length of reference sequence	100	88	100	100	100

Virulence factors play an important role in enhancing the ability of these organisms to cause illness. Virulence factors in the case of *Enterococcus* include extracellular proteins, bacteriocins, cell adhesion proteins, invasions, and intracellular proteins, and metabolic proteins. In general, although virulence-related genes are absent from our strains, some genes related to the above-mentioned factors are present in these strains; detail of each factor along with the number of genes are mentioned in Table 2. Virulence factor alone is not enough to explain the pathogenicity of Enterococcus as antimicrobial genes also have a major role in its pathogenicity.

The presence of genes related to transposons and mobile elements also contributes to increasing in pathogenicity of *Enterococcus,* as these mobile genetic elements transfer AR (antimicrobial genes) to nonpathogenic strains. This trans-conjugation

mechanism, by which they acquire AR resistance and even transfer of virulence factors, raises serious concern of using them as probiotics. Other genes related to antibiotics and toxic compounds were also seen in these isolates detail of which is incorporated in S3. Some important genes mentioned in S3 are related to resistance to Beta-lactamase, multidrug resistance efflux pump, resistance to cadmium, arsenic, and chromium compounds. Some genes related to fluoroquinolones were also present in both strains of *E. gallinrum*. The presence of genes related to resistance from fluoroquinolones shows presence of acquired gene resistance as described previously in many studies (Economou, Sakkas, Delis, & Gousia, 2017), (Jahan & Holley, 2016).

Table 5.7: Presence of different genes related to Antibiotic resistance and virulence factors in different strains of *enterococcus*.

***E. faecalis* QAU14**		
	Resistance to antibiotics and toxic compounds	66
1	Mercury resistance operon	4
2	Copper homeostasis	25
3	Bile hydrolysis	5
4	Resistance to fluoroquinolones	19
5	Beta-lactamase	2
6	Multidrug Resistance Efflux Pumps	3
7	Mercuric reductase	8
8	Virulence, Disease and Defense - no subcategory	0
***E. casseliflavus* QAU15**		
1	Resistance to antibiotics and toxic compounds	72
2	Copper homeostasis	24
3	Bile hydrolysis	2
4	Cobalt-zinc-cadmium resistance	19
5	Streptothricin resistance	1
6	Resistance to fluoroquinolones	5
7	Arsenic resistance	4
8	Fosfomycin resistance	2
9	Beta-lactamase	6
10	Cadmium resistance	4
11	Multidrug Resistance Efflux Pumps	4
12	Resistance to chromium compounds	1
	QAU16 / QAU17	
1	Virulence, Disease and Defense	60
2	Adhesion	1
3	Bacteriocins, ribosomally synthesized antibacterial peptides	9
4	Resistance to antibiotics and toxic compounds	35
5	Invasion and intracellular resistance	15

Additional analysis of plasmid-associated genes was also calculated using plasmid finder by RAST software. It did not show the presence of genes associated with plasmids, indicating that in these strains plasmid are not much involved in antibiotics resistance transfer genes, as plasmid have a central role in the transfer of resistance genes, but in place of plasmids mobile genetic elements are there which help in transfer of AR genes in strains.

5.4.4. Whole genome sequencing of predominant gram-negative isolates

Whole genome sequencing was carried out using long read sequencing technique. MinION results showed presence of different gram-negative isolates, whole genome sequence was studies showing presence of different virulence and antimicrobial genes.

5.4.4.1. *Klebsiella pneumoniae* QAU3, QAU5, QAU11, QAU12

K. pneumoniae is commonly found in host-associated niches and have very extensive phenotypic and genetic diversity. Most of previous studies reported a virulence plasmid that has an important role in making this bacterium more pathogenic. High throughput genomic analysis allows to probe and compare genetic diversity of isolated strains from human gut, helping to better understand pathogenesis of *K. pneumoniae.* Different strains of *K. pneumoniae*, when found in normal healthy amounts, helps us to digest carbohydrates such as lactose, resistant starches, inulin, fructose, and mannose. *K. pneumoniae* intestinal infections become problematic when the *K. pneumoniae* bacteria become opportunistic and increase in numbers.

An overgrowth of Klebsiella has been identified as a cause of bloating high starch diet, can trigger a growth of *K. pneumoniae* in the bowel, the starch becoming a main food supply for *K. pneumoniae*. Most common symptoms include abdominal pain, discomfort, bloating, constipation, diarrhea, flatulence, nausea, heartburn, reflux, headaches, joint and muscle pain, fatigue, weakness.

Table 5.8: Genome size and GC content of *K. Pneumonia*

Strains	Genome size	GC content	N50	L50	No. of Contigs	Number of Subsystems	Number of Coding Sequences	Number of RNAs
QAU3	4,665,450	55.4	4665187	1	2	375	5000	108
QAU5	5,202,186	50.5	5111949	1	13	372	5809	113
QAU11	11,679,63	52.3	593185	4	138	445	14322	248
QAU12	12,345,67	53.5	593185	4	138	666	12106	251

5.4.4.1.1. Genome size and GC Content

Genome size of *K. Pneumonia* varies greatly ranging from 4,665,450 to 12,345,67 having GC content above 50, table is attached for detail information. Looking deep into Pan genome analysis (sum of all core and accessory gene) show extreme divergence as more then 100,000 protein coding sequences are present. Most of these accessory genes are sporadic only present in 10% of genomes.

5.4.4.1.2. Antimicrobial resistance genes

Multidrug-resistance clones usually have resistance to ≥3 antimicrobial classes in addition to Ampicillin. In *K. Pneumonia* majority of antibiotic resistance is associated with horizontally acquired AMR genes. Resistance genes are non-uniformly distributed among population. Plasmid are an important source of transferring these genes.

In our samples, the majority of *Klebsiella pneumoniae* were multi-drug resistant (82%). The bacteria were found resistant to most β-lactam antibiotics, aminoglycosides, ciprofloxacin, cotrimoxazole, carbapenem, piperacillin, and tazobactam. Plasmid were also found in gene study of *K. pneumonia* , horizontal gene transfer is associated with plasmid carrying multiple resistance of genes. The presence of plasmid in these isolates also show presence of multi drug resistance in gut of IBS patients. Whole-genome sequencing (WGS) analyses of these isolates revealed diversity of resistance genes (see Table 5.10).

Amikacin encoding genes were also identified in majority of isolates. However, all isolates were shown to carry the blaCTX-M-15 ESBL gene, which could suggest clonal dissemination among these isolates. All four isolates have also shown to carry

.AmR genes among strains. Many plasmid are found in these strains number of each are given in **table.**

Most of strains were resistance to Amikacin, Amoxicillin/ clavulanate, Cefepime, Ceftriaxone, Ciprofloxin, Co-trimoxazole, Gentamicin, while genes responsible for these mutations were also observed by using CARD software, mostly Emr gene was present in all isolates. This is multidrug export protein and is part of tripartite efflux system EmrAB-TolC which confers resistance to commonly used antibiotics. Another important ARO gene CTX-M 15 was also commonly found in these strains, *ctx* gene is carried by plasmid and spreads between *Enterobacteriaceae* family. This gene is responsible for resistance to ceftazidime, cefotaxime, penicillin as well as to cephalosporins. An outer membrane efflux protein TolC was seen in most of strains, these efflux protein help in export of small molecules and toxins across membrane thus playing a major role in bacterial virulence. They act as important vehicle for export of plasmid and chromosomal associated toxins.

Table 5.9: Antibiotic susceptibility testing of *K. Pneumonia*

Antibiotics	**QAU 3**	**QAU 5**	**QAU 11**	**QAU 12**
Amikacin	R	S	R	R
Amoxicillin/ clavulanate	R	S	R	R
Cefepime	R	S	R	R
Ceftriaxone	R	R	R	R
Ciprofloxin	R	R	R	R
Co-trimoxazole	R	R	R	S
Gentamicin	R	S	S	S
Imipenem	S	S	S	S
Meropenem	S	S	S	S
Piperacillin+ Tazobactam	S	S	S	S

Table 5.10: Presence of different antimicrobial genes and class of drug resistance and their mechanism according to CARD based upon WGS data.

ARO	AMR gene family	Drug class	Resistance mechanism
emrY emrK emrR	major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline	antibiotic efflux
mdtE gadX cpxA	resistance-nodulation-cell division (RND) antibiotic efflux pump	macrolide, fluoroquinolone, penam	antibiotic efflux
CTX-M 15	CTX-M beta-lactamase	Cephalosporin	antibiotic inactivation
TolC	ATP-binding cassette (ABC) antibiotic efflux pump,	macrolide, fluoroquinolone aminoglycoside, carbapenem, cephalosporin,	antibiotic efflux

5.4.4.1.3. Pan genome analysis:

Klebsiella pneumonia sub strains analysis was done, result showed comparison of genetic diversity among different strains. By using G- view server we conclude that *K. pneumonia* has an open pan genome and a large population diversity, which means it have high genomic diversity as well.

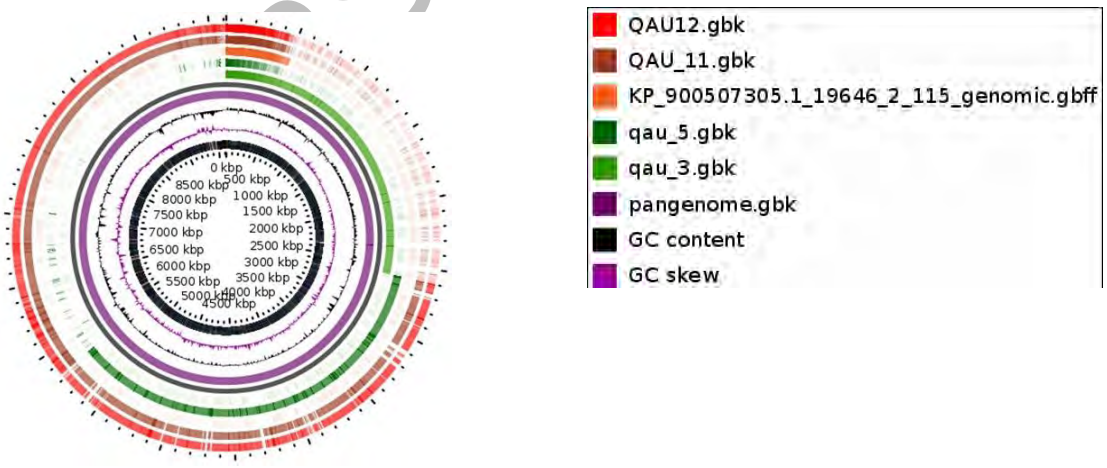


Figure 5.2: Circular map of *Klebsiella pneumonia* showing comparative genomicsbetween an already reported genome of *Klebsiella pneumonia* on NCBI.

Table 5.11: Genetic characterization of *Enterococcus* strains on basis of WGS results shows the presence of different genes related to its subsystem features.

Subsystem features	*K. pneumonia* QAU3	*K. pneumonia* QAU5	*K. pneumonia* QAU11	*K. pneumonia* QAU12
Cofactors, vitamins, prosthetic groups, pigments	175	175	398	175
Cell wall and capsule	44	44	119	44
Adhesion			11	
Toxins and superantigens			0	
Bacteriocins,			5	
Resistance to antibiotics and toxic compounds			96	
Virulence, disease	56	56	141	56
Invasion and intracellular resistance			29	
Potassium metabolism	17	17	37	13
Photosynthesis	0		-	106
Miscellaneous	38	38	85	54
Phages, prophages, transposable elements, plasmids	13	13	115	81
Membrane transport	106	106	350	109
Iron acquisition and metabolism	54	54	136	250
RNA Metabolism	81	81	156	9
Nucleosides and nucleotides	109	109	227	15
Protein metabolism	250	250	559	94
Cell division and cell cycle	9	9	18	28
Motility and chemotaxis	15	15	221	118
Regulation and cell signaling	94	94	192	61
Secondary metabolism	28	28	50	60
DNA metabolism	118	118	259	3
Fatty acids, lipids,	61	61	209	96
Nitrogen metabolism	60	60	128	108
Dormancy and sporulation	3	3	6	07
Respiration	96	96	256	347
Stress response	108	108	221	27
Aromatic compounds	07	07	105	30
Amino acids and derivatives	347	347	876	406
Sulfur metabolism	27	27	77	
Phosphorus metabolism	30	30	73	
Carbohydrates	406	406	1128	

5.4.4.2. *E. coli* QAU1, QAU2, QAU6, QAU7, QAU8, QAU9

Multidrug resistance in Escherichia coli has become a disquieting problem that is progressively observed in human. E. coli is now becoming inherently susceptible to almost all clinically available antimicrobial agents, most important mode of transferring resistance gene is by horizontal gene transfer. E.coli have high capability in acquiring genes for extended-spectrum β-lactamases (conferring resistance to broad-spectrum cephalosporins), carbapenemases (conferring resistance carbapenems), 16S rRNA methylases (conferring pan-resistance to aminoglycosides), plasmid-mediated quinolone resistance (PMQR) genes (conferring resistance to [fluoro]quinolones), and mcr genes (conferring resistance to polymyxins)

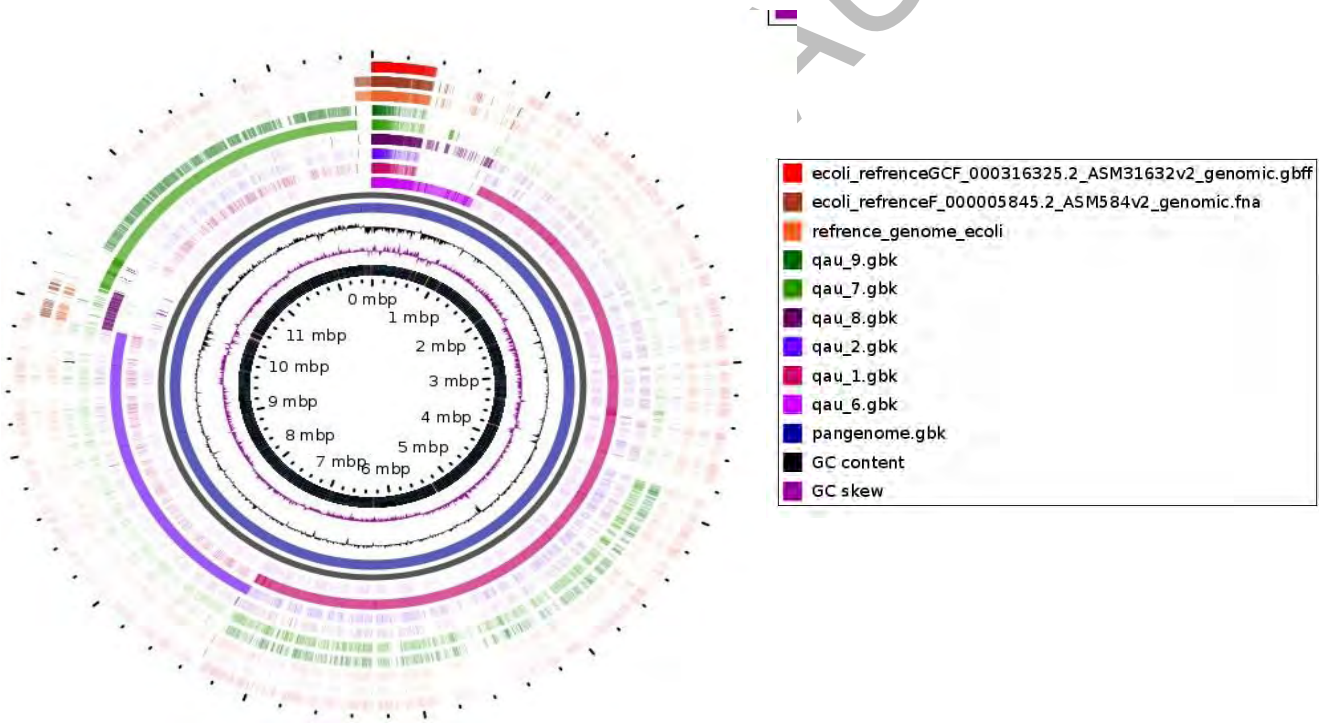


Figure 5.3: Circular map of *E.coli* showing comparative genomics between different species. QAU1, QAU2, QAU6, QAU7, QAU8 and QAU9 with an already reported genome of *E. coli* on NCBI.

E. coli associated with travelers' diarrhea and gut dysbiosis, Strains were enriched in virulence genes associated with extraintestinal pathogenic E. coli (ExPEC) and/or adherent-invasive *E.coli* are related to IBS symptoms like dysbiosis

Table 5.12: Genome details and GC content of various *E. Coli* strains

S.Nu	Genome size	GC content	N50	Number of Contigs	Number of Subsystems	Number of Coding Sequences	Number of RNAs
QAU1	10,266,006	53.6	4112988	23	428	12279	228
QAU2	5,013,446	55	-	1	567	5132	111
QAU6	5,220,996	50.4	5107798	2	601	5714	108
QAU7	5,853,255	56.9	5378609	7	394	6660	114
QAU8	4,985,384	50.8	4804817	4	604	5390	111

Table 5.13: Antibiotics data, commonly used antibiotics tested against different enterococcus strains using Khyber disk diffusion method.

Antibiotics	**QAU 1**	**QAU 2**	**QAU 6**	**QAU 7**	**QAU 8**	**QAU 9**
Amikacin	**R**	S	S	S	**R**	S
Amoxicillin	**R**	**R**	S	S	**R**	S
Ampicillin	**R**	**R**	S	S	**R**	S
Cefepime	**R**	**R**	S	S	**R**	S
Ceftriaxone	**R**	**R**	S	S	**R**	S
Cephradine	**R**	**R**	S	S	**R**	S
Ciprofloxin	**R**	S	S	S	**R**	S
Co-trimoxazole	**R**	S	S	S	**R**	S
Doxcycline	**R**	**R**	**R**	S	**R**	S
Gentamicin	**R**	S	**R**	**R**	S	S
Imipenem	S	S	**R**	**R**	S	S
Meropenem	S	S	**R**	**R**	S	S
Piperacillin+	**R**	S	**R**	**R**	S	S

Table 5.14: Presence of different antimicrobial genes and class of drug resistance and their mechanism according to CARD based upon WGS data.

ARO	AMR gene family	Drug class	Resistance mechanism
QnrB69	quinolone resistance protein (qnr)	Fluoroquinolone	antibiotic target protection
CRP BaeR	resistance-nodulation-cell division (RND) antibiotic efflux pump	macrolide, fluoroquinolone aminoglycoside, aminocoumarin	Antibiotic efflux
EmrR	major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone	Antibiotic efflux
EvgA	major facilitator superfamily (MFS) antibiotic efflux pump,	macrolide fluoroquinolone, tetracycline	Antibiotic efflux
MdtH	major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone	Antibiotic efflux

5.4.2.1. Antibiotic resistance in E.coli

E. coli strains vary depending upon gain or loss of virulences-associated traits. Number of genes found to be 4,000 to 5,000 in our isolates**.** Advanced studies in he genomic diversity of *E. coli* have been done using whole-genome sequencing, which give us detailed overweiw of virulence and antibiotic genes presenet, results are summerized in tables.

In our study genes related to carbapenemases have been detected in *E. coli,* also genes related to β-lactamases, AmpCs, and carbapenemases.were found in these isolates. Plasmids carrying ESBL genes are common among *E.coli*, we also found genes related to plasmid in our isolates.

Table 5.15: Genetic characterization of *E.coli* strains on basis of WGS results shows the presence of different genes related to its subsystem features.

Subsystem features	***E. coli* QAU1**	***E. coli* QAU2**	***E. coli* QAU8**	***E. coli* QAU9**
Cofactors, vitamins,	64	147	141	141
Cell wall and capsule	96	155	149	149
Adhesion	-	1	1	1
Toxins and superantigens	-	-	-	-
Bacteriocins,antibacterial peptides	-	13	9	9
Resistance to antibiotics and toxic compounds	66	72	-	-
Virulence, disease	-	-	-	-
Invasion	17	14	15	15
Potassium metabolism	-	11	10	10
Photosynthesis	-	-	-	-
Miscellaneous	4	34	19	19
Phages,	32	11	44	44
Membrane transport	57	99	84	***E. coli*** 84
Iron acquisition and metabolism	11	4	4	4
RNA Metabolism	57	137	120	120
Nucleosides and nucleotides	52	103	103	103
Protein metabolism	158	230	200	200
Cell division and cell cycle	7	54	42	42
Motility and chemotaxis	-	24	15	15
Regulation and cell signaling	24	44	41	41
Secondary metabolism	-	6	1	1
DNA metabolism	122	189	110	110
Fatty acids, lipids, and isoprenoids	4	99	75	75
Nitrogen metabolism	-	13	6	6
Dormancy and sporulation	-	4	2	2
Respiration	42	61	46	46
Stress response	40	77	60	60
Metabolism of aromatic compounds	-	2	2	2
Amino acids and derivatives	190	433	309	309
Sulfur metabolism	4	55	38	38
Phosphorus metabolism	23	32	33	33
Carbohydrates	331	859	657	657

5.4.4.3 *Shigella flexneri* QAU4

Shigellosis causative agents shigella a gram-negative facultative anaerobic bacteria was also prevalent in IBS patients because this is mainly associated with IBS-D causing symptoms. Also somtimes leads to acute mucosal inflammation, abdominal pain and bloody stools. So it can cause high ratio of mortality and morbidity, serology of shigella is dependent upon its O antigen present in its outer membrane(lipopolysacchride).

Although mostly in IBS antibiotic therapy is only choice left but Correct choice and dose of antibiotic play a vital role in reducing prevalence of infection, while its in correct oroverdose leads to drug resistance.

Diarrhea related symptoms were oftenly mistreated leading to drug resistance shigella spp.present strains of shigella are now resistance to even commoly used antibiotics which also effect cost and time of tretment.more than half of shigella worldwide are now resistance to multiple drugs, and these resistance mechanism limited options for shigella infection.present strain which we used as model to study antibiotic resistance in shigella is also resistance to many commonly used antibiotics.

5.4.3.1. Genome content and size of this strain

The **4,999,325**-bp bacterial chromosome of *S. flexneri* included 5600 coding sequences (CDS), 110 RNA sequences. The guanine and cytosine (GC) content of the genome found to account for 50.6 of total genome. A comparison of the genome features of QAU4 with other strains of *S. flexneri* showed a highly conserved composition and genome size (table 5.14). As mentioned in table:

Table 5.16: Genome details and GC content of *Shigella flexneri* QAU4

Strain	Genome size	GC content	N50	Number of Contigs	Number of Subsystems	Number of Coding Sequences	Number of RNAs
QAU4	4,999,325	50.6	4656065	30	362	5600	110

5.4.3.3. Pan genome analysis

Whole-genome alignment was done between QAU4 and publicly available strains of *S. flexneri,* results indicate a homologous genomic block common in two strains suggesting high similarity between strains. (fig. 2). GC content of QAU4 is overall conserved but results indicate considerable number of genomic shuffling and recombination events among different serotypes and strains of *S. flexneri*.

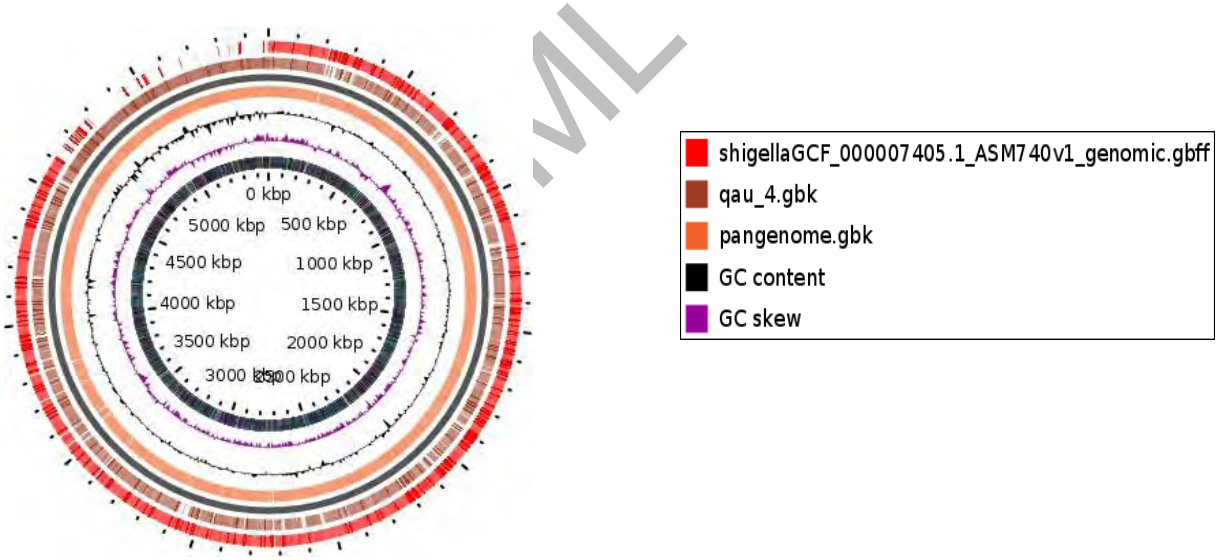


Figure 5.4: Circular map of S. flexneri with an already reported genome of S. flexneri on NCBI.

5.4.4.4. Antibiotic resistance

Earlier it was thought that Shigella pp were susceptible to ampicillin, chloramphenicol, co-trimoxazole and nalidixic acid but now they have developed resistance against fluoroquinolones, cephalosporins and azithromycin. Our phenotyphical results mentioned in table .Our study also shows same concern, shigella was shown to be resistance against most of common antibiotics like Amikacin, Amoxicillin, cefepime, cetriaxone, ciprofloxaxin, cotrimoxazole, gentamicin, and piperacillin only sesnsitive againts Imipenum and Meropenem.

Genetically when studied, presence of different antimicrobial genes was found which made this strain resistance to drugs. Resistance of *Shigella* spp.to chloramphenicol, streptomycin and tetracycline has mainly been attributed to the presence of *acrB, AcrE, AcrF* genes. Cephalosporin resistance gene CTX-M-15 was also identified while *emrA, Emrb, emrK, emrR* gene associated with fluoroquinolone antibiotic were also detected, these can be result of target site mutation as well as overexpression of genes encoding efflux pumps.

Table 5.17: Phenotypic data of commonly used antibiotics against shigella isolate.

Antibiotics	**Sensitivity**
Amikacin	**R**
Amoxicillin/ clavulanate	**R**
Cefepime	**R**
Ceftriaxone	**R**
Ciprofloxin	**R**
Co-trimoxazole	**R**
Gentamicin	**R**

Table 5.18: Presence of different antimicrobial genes and class of drug resistance and their mechanism according to CARD based upon WGS data.

ARO	AMR gene family	Drug class	Resistance mechanism
acrB, AcrE, AcrF	Resistance-nodulation-cell division (RND) antibiotic efflux pump	fluoroquinolone, cephalosporin, tetracyclin	Antibiotic efflux
CTX-M-15	CTX-M beta-lactamase	Cephalosporin	Antibiotic inactivation
bacA	proteins	peptide antibiotic	targ*et al*teration
emrA, Emrb,	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic	Antibiotic efflux
CRP	resistance-nodulation-cell division (RND)	Macrolide	Antibiotic efflux

5.4.4.3.*Proteus mirabilis* QAU10

An important member of Enterobacteriaceae family, living as commensals in gut is proteus, its previously thought to be only associated with UTI infections but now recently it was identified as potential pathogen in crohn's disease. Studies are now being conducted to study its role as gut pathogen, we also isolated this bacteria from gut of our IBS patients to study its virulence and genetic makeup.

Table 5.19: **Genome details and GC content of *Proteus mirabilis***

Strain	Genome size	GC content	N50	Number of Contigs	Subsystems	Number of Coding Sequences	Number of RNAs
QAU10	4,071,2	38.1	-	1	499	3376	110

5.4.3.1. Genome size

The **4,071,266**-bp bacterial chromosome of *proteus* included 3376 coding sequences (CDS), 110 RNA sequences. The guanine and cytosine (GC) content of the genome found to account for 38.1 of total genome. A comparison of the genome features of QAU10 with other strains showed a highly conserved composition and genome size .

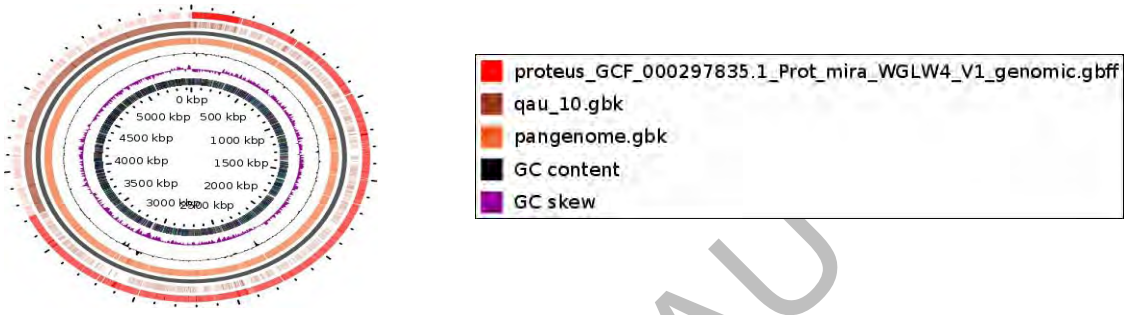


Figure 5.5: Circular map of *Proteus mirabilis* QAU10 with an already reported genome of on *Proteus mirabilis* NCBI.

5.4.3.2. Antibiotic resistance

Proteus species own many virulence factors hypothetically relevant to gastrointestinal pathogenicity, motility; adherence as well as production of urease, hemolysins, and IgA proteases; these all-increase the ability of proteus to acquire antibiotic resistance. Three types of resistance genes are detected in this isolate which make it resistance to commonly used antibiotics tetracycline, macrolide, fluroquinolone by making alteration in antibiotic efflux pump. Proteus species are thought to be have inherited resistance genes, most of these genes work by modification of lipids present in there membrane, present strain also have genes for conjugal transfer pilus, which helps in horizontal transfer of plasmid having antibiotic resistance genes.

Table 5.20: Antibiotics data, commonly used antibiotics tested against different enterococcus strains using Khyber disk diffusion method.

Antibiotics	Sensitivity
Amikacin	**R**
Amoxicillin/ clavulanate	**R**
Cefepime	**R**
Ceftriaxone	**R**
Ciprofloxin	**R**
Co-trimoxazole	**R**
Gentamicin	**R**
Imipenem	S
Meropenem	S
Piperacillin+ Tazobactam	S

Table 5.21: Presence of different antimicrobial genes and class of drug resistance and their mechanism according to CARD based upon WGS data.

ARO	AMR gene family	Drug class	Resistance mechanism
tetR	major facilitator superfamily (MFS) antibiotic efflux pump	tetracycline antibiotic	antibiotic target alteration, antibiotic efflux
CRP	resistance-nodulation-cell division (RND) antibiotic efflux pump	macrolide, fluoroquinolone, penam	Antibiotic efflux
EFT-u	elfamycin resistant EF-Tu	elfamycin antibiotic	Antibiotic target alteration

5.4. Discussion

Vancomycin and linezolid are the most commonly used drugs against *Enterococcus* in hospitals. Vancomycin-resistant organism (VRE) is known to cause serious infections that cannot be treated with common antibiotics. Treatment of VRE is a real challenge to clinicians, as vancomycin is usually the drug of last choice in treatment of enterococcal infections. Vancomycin is used as a replacement for penicillin, ampicillin, and aminoglycosides in patients with allergies (Franz & Holzapfel, 2004). In our isolates, we found resistance genes against vancomycin only in *E. gallinarum*

QAU17 and *E. casseliflavus* QAU15. *E. faecalis* QAU14 and *E. gallinarum* QAU16 were also resistant against Linezolid, which concludes the presence of 23S rRNA mutations and horizontally acquired resistance genes cfr and optrA. Three chromosomally located clustered genes *vanC1*, *vanXYC*, and *vanRC* were detected in these strains except *E. faecalis* but always there is a chance that these resistance genes can be transmissible to other bacteria. Therefore, it is suggested that more research is needed to study *vanC1, vanXYC*, and *vanRC* in *Enterococcus* strains as they are the reservoir for antimicrobial resistance genes. The WGS analyses proved that *E. faecalis, E. casseliflavus, E. gallinarum* isolates are not promising candidates for probiotics as they have antibiotic resistance and virulence genes.The gastrointestinal microbial flora usually presents in relationship of symbiosis or "commensalisms, but dysbiosis in gut environment leads to change this environment of bacterial community. This dysbiosis is often more common in IBS, one of study showed 73% IBS patients have gut dysbiosis (Karantanos *et al*., 2010; Jeffery *et al*., 2012) gram negative ratio increases as dysbiosis increases especially Proteobacteria such as Shigella or Escherichia; and Actinobacteria. Bacterioides stercoris and Bifidobacterium were reported to be higher in IBS leading to dysbiosis.

Past studies also show increase in prevalence of Pseudomonas aeruginosa in IBS patients (Kerckhoffs *et al*., 2011; Shukla *et al*., 2015), some other studies reported high levels of Enterobacteriaceae, Bacteroidetes such as Ruminococcus sp., Lactobacilli sp., and Clostridium sp. in the IBS patients (Si *et al*., 2004; Malinen *et al*., 2010; Ponnusamy *et al*., 2011).Gram negatives like E.coli, proteus and shigella increases in IBS patients and study of their genetic diversity reveled presence of antimicrobial genes in them. Gram negative bacteria develops natural ways to resist antimicrobial drugs mainly by preventing drugs from being absorbed or sometimes by reducing affinity of dugs to target cells. Sometimes due to modification of its cell wall(bacteria can modulate its cell wall) permeability changes, which can leads to increase in minimum inhibitory concentration(MIC) for antimicrobial agents.

One of common example use of quinolone antibacterial agents, such as nalidixic acid, ofloxacin, and ciprofloxacin, that works by interfering DNA replication. Same is case with use of Aminoglycoside antibiotics, such as streptomycin and spectinomycin, which works by inhibiting protein synthesis. β-Lactam antibiotics, eg, penicillin and

cephalosporin, most widely use against gram negatives works by targeting penicillin binding proteins, any mutation or change in penicillin-binding protein makes gram negative antimicrobial resistance. Most of resistance toward β-lactam antibiotics is associated with modification of the outer-membrane porins OmpF (~38 kDa) and OmpC (~42 kDa) and cytosolic proteins of ~26 kDa, OmpR as a transcriptional regulator. In our study most of gram negatives (shigella spp) were found resistance to imipenem which can be related to colicin resistance.

Escherichia coli, another important gram-negative isolates from gut is becoming multidrug resistance and have great capacity for acquisition of genes coding for extended-spectrum β-lactamases (conferring resistance to broad-spectrum cephalosporins), carbapenemases (conferring resistance to carbapenems), 16S rRNA methylases (conferring pan-resistance to aminoglycosides), plasmid-mediated quinolone resistance (PMQR) genes (conferring resistance to [fluoro]quinolones), and mcr genes (conferring resistance to polymyxins). Another important isolate from our IBS patients were Proteus spp, proteus are potentially pathogenic resident of human GIT tract and are known to cause gastrointestinal diseases. There is not much study on microbe and host interaction of proteus in gut however role of proteus in urinary track and intestinal dysbiosis suggest that it should be examined as potential pathogenic gastrointestinal microbe.

Our study revealed presence of virulence and antimicrobial resistance genes in proteus, so there is an increased chanced that they have a role in inflammatory bowel disease, production of lipopolysaccharide and flagellin protein also show this association (IBD Vs Proteus growth in gut). Study of antimicrobial sensitivity profile of proteus spp also suggests their relevance to pathogenicity in gut.

Conclusion

More comprehensive studies on metabolic pathways are needed to further evaluate the probiotic role of *Enterococcus*. Moreover, we used Oxford Nanopore technology MinION, which is proven to be less time-consuming and is cost-effective for screening normal gut flora of humans. The influence of commensal microbiota in the pathophysiology of IBS is important to understand because Gut dysbiosis is related virulent strains, Normal gut microbiota can cause dysbiosis due to presence of virulent genes. We were also able to describe some important characteristics of the

microbial community, including the strain diversity, the growth dynamics, and the presence of genes involved in bacterial virulence and in antibiotic resistance mechanisms that can provide an adaptive advantage to opportunistic and pathogenic microbes. Culturable microbiota isolated from IBS was divided into gram positive and negative groups and further characterization was carried out.

Gram negatives shows presence of various virulence gene which can make them pathogenic in gut environment leading to worsen IBS symptoms or some time to leaky gut. In most of strains resistance genes to commonly used antibiotics like mdtE,emrY, CRP were found.

Vancomycin and linezolid are most commonly used drugs against *Enterococcus* in hospitals,.Vancomycin resistance organism(VRE) are know to cause serious infections that could not be treated with common antibiotics. In our isolates we find out resistance genes against vancomycin only while two of *Enterococcus* strains *E. gallinarum* QAU17 and *E. casseliflavus* QAU15. *E. faecalis* QAU14 and *E. gallinarum* QAU16 are also resistance against Linezolid, which conclude presence of 23S rRNA mutations and horizontally acquired resistance genes cfr and optrA. Three chromosomally located clustered genes *vanC1*, *vanXYC*, and *vanRC* were detected in these strains except *E. faecalis* but always there is a chance that these resistance genes can be transmissible to other bacteria therefore it is suggested that more research is needed to study *vanC1, vanXYC*, and *vanRC* in *Enterococcus* strains as they are reservoir for antimicrobial resistance genes. The WGS analyses approved that *E. faecalis, E. casseliflavus, E. gallinarum* isolates are not promising candidates for probiotic as they have antibiotic resistance and virulence genes, more comprehensive studies leading towards metabolic pathways are needed to be further evaluate *Enterococcus* role as probiotic.

Conclusions

In Pakistani population this disease is present in all age groups. All three types of IBS were prevalent among selected samples; however IBSM was more than IBS C and IBS D. About half of the IBS patients were depressed, and strong correlation wasfound between IBS severity score and depression. There was a strong correlation between serum vitamin-D and butyrate.

Psychosocial stress likely alters gut microbiota, increases mucosal permeability, motility, and induces visceral hyperalgesia in IBS patients. The production of butyrate decreases during the curse of IBS suggesting ratio of butyrate production bacteria decreases in IBS. Vitamin D deficiency was very high in IBS patients (82%), which can be dueto lower number of firmicutes ratio in IBS patients. IBS itself could cause elevated liver enzymes, but its not much prominent inIBS. The lipid-lowering effects of intestinal bacteria, such as specific strains of Lactobacillus or Bifidobacterium is lower in IBS patients due to which lipid profiling got disturbed. our study proposes a comprehensive characterization of the microbiome in IBS, the use of metagenomic sequencing data allowed us to explore the complexity of the gut microbial ecosystem with high resolution. Pathophysiology of IBS is corelated with some physiological parameters like vitamin D, butyrate and depression levels, so butyrate and vitamin D level can be an important biomarker for IBS detection.

In IBS patients gut microbiota diversity was lower, so Poor diversity and gut dysbiosis have significant impact on progression of IBS in patients having more severity symptoms. Ratio to opportunistic pathogens were high especially members of enterobacteriaceace, few microbial groups like weissella, ruminococcus, enterobacteriaceae have prominent impact in low vitamin D and high depression level in patients. Virulent genes makes normal gut flora as pathogenic leading to leaky gut or gut inflammation

Future Prospects

- As it was a pilot study so future studies require more data from different regions of country to have more understanding about pathophysiology of IBS.
- Although we study genes involved in IBS progression, but more comprehensive study related to Gut neuromuscular dysfunction needs to design which will help in understanding disease mechanism.
- Brain–gut pathways in progression of IBS needs to be studied.
- Management of IBS to lower symptoms severity in IBS.
- Meta-transcriptomics and metabolomics data, as well as functional experiments on IBS is required to know the pathophysiology of disease complete.

References

Abd El-Baky, R. M., Ibrahim, R. A., Mohamed, D. S., Ahmed, E. F., & Hashem, Z. S. (2020). Prevalence of virulence genes and their association with antimicrobial resistance among pathogenic E. coli isolated from Egyptian patients with different clinical infections. *Infection and DrugResistance, 13*, 1221.

Alcock, B. P., Raphenya, A. R., Lau, T. T., Tsang, K. K., Bouchard, M., Edalatmand, A., Liu, S. (2020). CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic acids research, 48*(D1), D517-D525.

Ameur, A., Kloosterman, W. P., & Hestand, M. S. (2019). Single-molecule sequencing: towards clinical applications. *Trends in biotechnology, 37*(1), 72-85.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., . . . Kubal, M. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC genomics, 9*(1), 1-15.

Angelakis, E., *et al*. (2013). "Related actions of probiotics and antibiotics on gut microbiota and weight modification." The Lancet infectious diseases **13**(10): 889-899.

Atluri, D., *et al*. (2014). "Effect of linaclotide in irritable bowel syndrome with constipation (IBS- C): a systematic review and meta- analysis." Neurogastroenterology & Motility **26**(4): 499- 509.

Abubucker, S., *et al*. (2012). "Metabolic reconstruction for metagenomic data and its application to the human microbiome." PLoS computational biology **8**(6).

Adhyaru, B. B. and T. A. Jacobson (2016). "New cholesterol guidelines for the management of atherosclerotic cardiovascular disease risk: a comparison of the 2013 American College of Cardiology/American Heart Association cholesterol guidelines with the 2014 National Lipid Association recommendations for patient-centered management of dyslipidemia." Endocrinology and Metabolism Clinics **45**(1): 17-37.

Agarwal, N. and B. M. Spiegel (2011). "The effect of irritable bowel syndrome on health-related quality of life and health care expenditures." Gastroenterology Clinics **40**(1): 11-19.

Agrawal, A. and P. Whorwell (2006). "Irritable bowel syndrome: diagnosis and management." Bmj **332**(7536): 280-283.

Biyyani, R. S. R. S., *et al*. (2010). "Dyslipidemia and lipoprotein profiles in patients with inflammatory bowel disease." Journal of clinical lipidology **4**(6): 478-482.

Barbalho, S. M., *et al*. (2019). "Irritable bowel syndrome: a review of the general aspects and thepotential role of vitamin D." Expert review of gastroenterology & hepatology **13**(4): 345-359.

Bjørneklett, A. and E. Jenssen (1982). "Relationships between hydrogen (H2) and methane (CH4) production in man." Scandinavian journal of gastroenterology **17**(8): 985-992.

Bolyen, E., *et al*. (2018). QIIME 2: Reproducible, interactive, scalable, and extensible microbiomedata science, PeerJ Preprints.

Bai, Y., *et al*. (2019). "Cinobufacini ameliorates experimental colitis via modulating thecomposition of gut microbiota." PloS one **14**(9).

Barcenilla, A., *et al*. (2000). "Phylogenetic relationships of butyrate-producing bacteria from thehuman gut." Appl. Environ. Microbiol. **66**(4): 1654-1661.

Bron, P. A., *et al*. (2017). "Can probiotics modulate human disease by impacting intestinal barrierfunction?" British Journal of Nutrition **117**(1): 93-107.

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., & Harwood, V. J. (2012). Enterococci in the environment. *Microbiology and Molecular Biology Reviews, 76*(4), 685-706.

Barbara, G., *et al*. (2019). "Rome Foundation Working Team Report on post-infection irritablebowel syndrome." Gastroenterology **156**(1): 46-58. e47.

Biasucci, G., *et al*. (2010). "Mode of delivery affects the bacterial community in the newborn gut."Early human development **86**(1): 13-15.

Castro-Wallace, S. L., Chiu, C. Y., John, K. K., Stahl, S. E., Rubins, K. H., McIntyre, A. B., Botkin, D. J. (2017). Nanopore DNA sequencing and genome assembly on the International Space Station. *Scientific reports, 7*(1), 1-12.

Camilleri, M., *et al*. (2014). "Effect of increased bile acid synthesis or fecal excretion in irritable bowel syndrome-diarrhea." The American journal of gastroenterology **109**(10): 1621.

Camilleri, M., *et al*. (2008). "Genetic variation in endocannabinoid metabolism, gastrointestinal motility, and sensation." American Journal of Physiology-Gastrointestinal and Liver Physiology **294**(1): G13-G19.

Canavan, C., *et al*. (2014). "The epidemiology of irritable bowel syndrome." Clinical epidemiology**6**: 71.

Carabotti, M., *et al*. (2015). "The gut-brain axis: interactions between enteric microbiota, central andenteric nervous systems." Annals of Gastroenterology: Quarterly Publication of the Hellenic Societyof Gastroenterology **28**(2): 203.

Chang, P. V., *et al*. (2014). "The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition." Proceedings of the National Academy of Sciences **111**(6): 2247-2252.

Cole, J. A., *et al*. (2006). "Migraine, fibromyalgia, and depression among people with IBS: a prevalence study." BMC gastroenterology **6**(1): 26.

Cremon, C., *et al*. (2009). "Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms." The American journal of gastroenterology **104**(2): 392.

Cremonini, F. and N. J. Talley (2005). "Irritable bowel syndrome: epidemiology, natural history, health care seeking and emerging risk factors." Gastroenterology Clinics **34**(2): 189-204.

Carroll, I. M., *et al*. (2010). "Luminal and mucosal-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome." Gut pathogens **2**(1): 19

Carroll, I. M., *et al*. (2012). "Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea- predominant irritable bowel syndrome." Neurogastroenterology & Motility **24**(6): 521-e248.

Chung, C.-S., *et al*. (2016). "Differences of microbiota in small bowel and faeces between irritable bowel syndrome patients and healthy subjects." Scandinavian journal of gastroenterology **51**(4): 410-419.

Collins, S. (2016). The intestinal microbiota in the irritable bowel syndrome. International review ofneurobiology, Elsevier. **131:** 247-261.

Caporaso, J. G., *et al*. (2010). "PyNAST: a flexible tool for aligning sequences to a template alignment." Bioinformatics **26**(2): 266-267.

Caporaso, J. G., *et al*. (2010). "QIIME allows analysis of high-throughput community sequencing data." Nature methods **7**(5): 335.

Carroll, I. M., *et al*. (2010). "Luminal and mucosal-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome." Gut pathogens **2**(1): 19.

Carroll, I. M., *et al*. (2011). "Molecular analysis of the luminal-and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome." American Journal of Physiology- Gastrointestinal and Liver Physiology **301**(5): G799-G807.

Catanzaro, R., *et al*. (2014). "Irritable bowel syndrome: new findings in pathophysiological and therapeutic field." Minerva gastroenterologica e dietologica **60**(2): 151-163.

Cantu-Jungles, T. M., *et al*. (2017). "A pectic polysaccharide from peach palm fruits (Bactris gasipaes) and its fermentation profile by the human gut microbiota in vitro." Bioactive Carbohydrates and Dietary Fibre **9**: 1-6.

Canavan, C., *et al*. (2014). "The epidemiology of irritable bowel syndrome." Clinical epidemiology **6**: 71.

Chang, F.-Y., *et al*. (2010). "The current prevalence of irritable bowel syndrome in Asia." Journal ofneurogastroenterology and motility **16**(4): 389.

Conlon, M. A. and A. R. Bird (2015). "The impact of diet and lifestyle on gut microbiota and human health." Nutrients **7**(1): 17-44.

Cremonini, F. and N. J. Talley (2005). "Irritable bowel syndrome: epidemiology, natural history, health care seeking and emerging risk factors." Gastroenterology Clinics **34**(2): 189-204.

Drossman, D. A. (2016). "Functional gastrointestinal disorders: history, pathophysiology, clinical features, and Rome IV." Gastroenterology **150**(6): 1262-1279. e1262.

Drossman, D. A., *et al*. (2000). "Functional bowel disorders and functional abdominal pain." The functional gastrointestinal disorders.

Duncan, S. H., *et al*. (2002). "Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces." International journal of systematic and evolutionary microbiology **52**(5): 1615-1620.

De Filippo, C., *et al*. (2010). "Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa." Proceedings of the National Academy of Sciences **107**(33): 14691-14696.

Duncan, S. H., *et al*. (2002). "Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces." International journal of systematic and evolutionary microbiology **52**(5): 1615-1620.

D'Aversa, F., *et al*. (2013). "Gut microbiota and metabolic syndrome." Internal and emergency medicine **8**(1): 11-15.

Dupont, H. (2014). "evidence for the role of gut microbiota in irritable bowel syndrome and its potential influence on therapeutic targets." Alimentary pharmacology & therapeutics **39**(10): 1033- 1042.

Drossman, D. A., *et al*. (2000). "Further validation of the IBS-QOL: a disease-specific quality-of- life questionnaire." The American journal of gastroenterology **95**(4): 999-1007.

Dishman, R. K., *et al*. (2006). "Neurobiology of exercise." Obesity **14**(3): 345-356.

Edgar, R. C. (2010). "Search and clustering orders of magnitude faster than BLAST." Bioinformatics **26**(19): 2460-2461.

El-Salhy, M., *et al*. (2013). "The role of peptide YY in gastrointestinal diseases and disorders." International journal of molecular medicine **31**(2): 275-282.

Elangovan, S., *et al*. (2014). "The niacin/butyrate receptor GPR109A suppresses mammary tumorigenesis by inhibiting cell survival." Cancer research **74**(4): 1166-1178.

El Amrousy, D., *et al*. (2018). "Vitamin D supplementation in adolescents with irritable bowel syndrome: Is it useful? A randomized controlled trial." Saudi journal of gastroenterology: official journal of the Saudi Gastroenterology Association **24**(2): 109.

Economou, V., Sakkas, H., Delis, G., & Gousia, P. (2017). Antibiotic resistance in Enterococcus spp. Friend or foe? *Foodborne Pathogens and Antibiotic resistance, ed. OM Singh (Hoboken, NJ: John Wiley & Sons)*.

Farup, P. G., *et al*. (2016). "Faecal short-chain fatty acids-a diagnostic biomarker for irritable bowelsyndrome?" BMC gastroenterology **16**(1): 51.

Fichna, J. and M. Storr (2012). "Brain-gut interactions in IBS." Frontiers in pharmacology **3**: 127

Faith, J. J., *et al*. (2013). "The long-term stability of the human gut microbiota." Science **341**(6141):1237439.

Fukuda, S., *et al*. (2011). "Bifidobacteria can protect from enteropathogenic infection throughproduction of acetate." Nature **469**(7331): 543-547.

Franz, C., & Holzapfel, W. H. (2004). The genus Enterococcus: biotechnological and safety issues. *Food science and technology-new york-marcel dekker-, 139*, 199-248.

Fond, G., *et al*. (2014). "Anxiety and depression comorbidities in irritable bowel syndrome (IBS): a systematic review and meta-analysis." European archives of psychiatry and clinical neuroscience **264**(8): 651-660.

Ghoshal, U. C., *et al*. (2017). "Small intestinal bacterial overgrowth and irritable bowel syndrome: abridge between functional organic dichotomy." Gut and liver **11**(2): 196.

Gwee, K. A., *et al*. (2010). "Asian consensus on irritable bowel syndrome." Journal of gastroenterology and hepatology **25**(7): 1189-1205.

Giamarellos-Bourboulis, E., *et al*. (2015). "Molecular assessment of differences in the duodenal microbiome in subjects with irritable bowel syndrome." Scandinavian journal of gastroenterology **50**(9): 1076-1087.

Goldman, B., *et al*. (2006). "Evolution of sensory complexity recorded in a myxobacterial genome."Proceedings of the National Academy of Sciences **103**(41): 15200-15205.

Guilera, M., *et al*. (2005). "Bowel habit subtypes and temporal patterns in irritable bowel syndrome:systematic review." The American journal of gastroenterology **100**(5): 1174.

Ghoshal, U. C., *et al*. (2013). "Comparison of Manning, Rome I, II, and III, and Asian diagnostic criteria: report of the Multicentric Indian Irritable Bowel Syndrome (MIIBS) study." Indian Journal of Gastroenterology **32**(6): 369-375.

Gwee, K. A., *et al*. (2019). "Second asian consensus on irritable bowel syndrome." Journal of neurogastroenterology and motility **25**(3): 343.

Gwee, K. A., *et al*. (2009). "Epidemiology of irritable bowel syndrome in Asia: something old, something new, something borrowed." Journal of gastroenterology and hepatology **24**(10): 1601- 1607.

Herring, M. P., *et al*. (2012). "Effect of exercise training on depressive symptoms among patients with a chronic illness: a systematic review and meta-analysis of randomized controlled trials." Archives of Internal Medicine **172**(2): 101-111.

Hayashi, H., *et al*. (2002). "Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture- based methods." Microbiology and

immunology **46**(8): 535-548.

Hu, S., *et al*. (2019). "Gut microbiota changes in patients with bipolar depression." Advanced Science **6**(14): 1900752.

Hatayama, H., *et al*. (2007). "The short chain fatty acid, butyrate, stimulates MUC2 mucin production in the human colon cancer cell line, LS174T." Biochemical and biophysical research communications **356**(3): 599-603.

Hollister, E. B., *et al*. (2020). "Relationships of microbiome markers with extraintestinal, psychological distress and gastrointestinal symptoms, and quality of life in women with irritable bowel syndrome." Journal of clinical gastroenterology **54**(2): 175-183.

Haney, E. F., Mansour, S. C., & Hancock, R. E. (2017). Antimicrobial peptides: an Introduction to *Antimicrobial Peptides*, 3-22.

Hazaa, H. F. and F. H. Lami (2018). "Irritable bowel syndrome among High School Students, Bagdad, Iraq, 2017-2018." Journal of the Faculty of Medicine Baghdad **60**(2): 113-118.

Jahan, M., & Holley, R. (2016). Transfer of antibiotic resistance from Enterococcus faecium of fermented meat origin to Listeria monocytogenes and Listeria innocua. *Letters in applied microbiology, 62*(4), 304-310.

Jafri, W., *et al*. (2007). "Irritable bowel syndrome and health seeking behaviour in different communities of Pakistan." Journal of Pakistan Medical Association **57**(6): 285.

Jalanka-Tuovinen, J., *et al*. (2014). "Faecal microbiota composition and host–microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome." Gut **63**(11): 1737-1745.

Jalanka-Tuovinen, J., *et al*. (2014). "Faecal microbiota composition and host–microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome." Gut **63**(11): 1737-1745.

Johannesson, E., *et al*. (2015). "Intervention to increase physical activity in irritable bowel syndrome shows long-term positive effects." World journal of gastroenterology: WJG **21**(2): 600.

Jeffery, I. B., *et al*. (2012). "An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota." Gut **61**(7): 997-1006.

Jiang, H., *et al*. (2015). "Altered fecal microbiota composition in patients with major depressivedisorder." Brain, behavior, and immunity **48**: 186-194.

Kalantar-Zadeh, K., *et al*. (2019). "Intestinal gases: influence on gut disorders and the role of dietarymanipulations." Nature reviews Gastroenterology & hepatology: 1-15.

Kang, S. S., *et al*. (2014). "Diet and exercise orthogonally alter the gut microbiome and reveal independent associations with anxiety and cognition." Molecular neurodegeneration **9**(1): 36.

Kerckhoffs, A. P., *et al*. (2009). "Lower Bifidobacteria counts in both duodenal mucosa-associated and fecal microbiota in irritable bowel syndrome patients." World journal of gastroenterology: WJG**15**(23): 2887.

Kuczynski, J., *et al*. (2011). "Using QIIME to analyze 16S rRNA gene sequences from microbial communities." Current protocols in bioinformatics **36**(1): 10.17. 11-10.17. 20..

Krogius-Kurikka, L., *et al*. (2009). "Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers." BMC gastroenterology **9**(1): 95.

Koppen, I. J., *et al*. (2017). "The pediatric Rome IV criteria: what's new?" Expert review of gastroenterology & hepatology **11**(3): 193-201.

Lozupone, C. A., *et al*. (2012). "Diversity, stability and resilience of the human gut microbiota." Nature **489**(7415): 220.

Labus, J. S., *et al*. (2017). "Differences in gut microbial composition correlate with regional brain volumes in irritable bowel syndrome." Microbiome **5**(1): 49.

Leite, G. G., *et al*. (2020). "Mapping the Segmental Microbiomes in the Human Small Bowel in Comparison with Stool: A REIMAGINE Study." Digestive diseases and sciences: 1-10.

Liu, Y., *et al*. (2016). "Similar fecal microbiota signatures in patients with diarrhea-predominant irritable bowel syndrome and patients with depression." Clinical Gastroenterology and Hepatology **14**(11): 1602-1611. e1605.

Labus, J. S., *et al*. (2017). "Differences in gut microbial composition correlate with regional brain volumes in irritable bowel syndrome." Microbiome **5**(1): 49.

Lagier, J.-C., *et al*. (2016). "Culture of previously uncultured members of the human gut microbiota by culturomics." Nature microbiology **1**(12): 1-8.

Lepp, P. W., *et al*. (2004). "Methanogenic Archaea and human periodontal disease." Proceedings of the National Academy of Sciences **101**(16): 6176-6181.

Liu, Y., *et al*. (2016). "Similar fecal microbiota signatures in patients with diarrhea-predominant irritable bowel syndrome and patients with depression." Clinical Gastroenterology and Hepatology **14**(11): 1602-1611. e1605.

Lozupone, C. A., *et al*. (2012). "Diversity, stability and resilience of the human gut microbiota." Nature **489**(7415): 220-230.

Lovell, R. M. and A. C. Ford (2012). "Prevalence of gastro-esophageal reflux-type symptoms in individuals with irritable bowel syndrome in the community: a meta-analysis." The American journal of gastroenterology **107**(12): 1793.

Liang, C.-M., Hsu, C.-H., Chung, C.-H., Chen, C.-Y., Wang, L.-Y., Hsu, S.-D., . . . Hu, J.-M. (2020). Risk for Irritable Bowel Syndrome in Patients with Helicobacter Pylori Infection: A

Nationwide Population-Based Study Cohort Study in Taiwan. *International Journal of Environmental Research and Public Health, 17*(10), 3737.

Loh, G., & Blaut, M. (2012). Role of commensal gut bacteria in inflammatory bowel diseases. *Gut Microbes, 3*(6), 544-555.

Lovell, R. M. and A. C. Ford (2012). "Effect of gender on prevalence of irritable bowel syndrome in the community: systematic review and meta-analysis." American Journal of Gastroenterology **107**(7): 991-1000.

Malinen, E., *et al*. (2005). "Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR." American Journal of Gastroenterology **100**(2): 373-382.

Manichanh, C., *et al*. (2014). "Anal gas evacuation and colonic microbiota in patients with flatulence: effect of diet." Gut **63**(3): 401-408.

Masella, A. P., *et al*. (2012). "PANDAseq: paired-end assembler for illumina sequences." BMCbioinformatics **13**(1):

Maukonen, J., *et al*. (2006). "Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria." Journal of medical microbiology **55**(5): 625-633.

Moore, W., *et al*. (1972). "Ruminococcus bromii sp. n. and emendation of the description of Ruminococcus Sijpestein." International journal of systematic and evolutionary microbiology **22**(2):78-80.

Maharshak, N., *et al*. (2018). "Fecal and mucosa-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome." Digestive diseases and sciences **63**(7): 1890-1899. Manichanh, C., *et al*. (2014). "Anal gas evacuation and colonic microbiota in patients with flatulence: effect of diet." Gut **63**(3): 401-408.

Maukonen, J., *et al*. (2006). "Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria." Journal of medical microbiology **55**(5): 625-633.

Menees, S. and W. Chey (2018). "The gut microbiome and irritable bowel syndrome." F1000Research **7**.

Martínez, I., *et al*. (2010). "Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects." PloS one **5**(11): e15046.

Mazzawi, T., *et al*. (2015). "Effect of dietary management on the gastric endocrine cells in patients with irritable bowel syndrome." European journal of clinical nutrition **69**(4): 519.

McNabney, S. and T. Henagan (2017). "Short chain fatty acids in the colon and peripheral tissues: a focus on butyrate, colon cancer, obesity and insulin resistance." Nutrients **9**(12): 1348.

Mertz, H., *et al*. (2000). "Regional cerebral activation in irritable bowel syndrome and control subjects with painful and nonpainful rectal distention." Gastroenterology **118**(5):

842-848.

Moens, F., *et al*. (2016). "Bifidobacterial inulin-type fructan degradation capacity determines cross- feeding interactions between bifidobacteria and Faecalibacterium prausnitzii." International journal of food microbiology **231**: 76-85.

Monsbakken, K., *et al*. (2006). "Perceived food intolerance in subjects with irritable bowel syndrome–etiology, prevalence and consequences." European journal of clinical nutrition **60**(5): 667.

Nagel, R., *et al*. (2016). "Comparison of faecal microbiota in Blastocystis-positive and Blastocystis- negative irritable bowel syndrome patients." Microbiome **4**(1): 47.

Noor, S. O., *et al*. (2010). "Ulcerative colitis and irritable bowel patients exhibit distinct abnormalities of the gut microbiota." BMC gastroenterology **10**(1): 134.

Nwosu, B. U., *et al*. (2017). "Vitamin D status in pediatric irritable bowel syndrome." PloS one **12**(2).

Naeem, S. S., *et al*. (2012). "Prevalence and factors associated with irritable bowel syndrome among medical students of Karachi, Pakistan: A cross-sectional study." BMC Research Notes **5**(1): 255.

Ozanne, G., Mathieu, L., & Baril, J. (1977). Production of colicin V in vitro and in vivo andobservations on its effects in experimental animals. *Infection and immunity, 17*(3), 497-503.

Ohkusa, T. (2019). "Chronic Constipation, Gut Microbiota and New treatment for constipation."Frontiers in Medicine **6**: 19.

Odamaki, T., *et al*. (2016). "Age-related changes in gut microbiota composition from newborn tocentenarian: a cross-sectional study." BMC microbiology **16**(1): 90.

O'sullivan, M., *et al*. (2000). "Increased mast cells in the irritable bowel syndrome." Neurogastroenterology and Motility **12**(5): 449-458.

Posserud, I., *et al*. (2007). "Altered rectal perception in irritable bowel syndrome is associated withsymptom severity." Gastroenterology **133**(4): 1113-1123.

Power, S. E., *et al*. (2014). "Intestinal microbiota, diet and health." British Journal of Nutrition **111**(3): 387-402.

Pace, F., *et al*. (2015). "Probiotics in digestive diseases: focus on Lactobacillus GG." Minervagastroenterologica e dietologica **61**(4): 273-292.

Parkes, G., *et al*. (2012). "Distinct microbial populations exist in the mucosa- associated microbiota of sub- groups of irritable bowel syndrome." Neurogastroenterology & Motility **24**(1): 31-39.

Peng, L., *et al*. (2007). "Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier." Pediatric research **61**(1): 37-41.

Parkes, G. C., Brostoff, J., Whelan, K., & Sanderson, J. D. (2008). Gastrointestinal microbiota in irritable bowel syndrome: their role in its pathogenesis and treatment. *American Journal of Gastroenterology, 103*(6), 1557-1567.

Pace, F., *et al*. (2015). "Probiotics in digestive diseases: focus on Lactobacillus GG." Minerva gastroenterologica e dietologica **61**(4): 273-292.

Parkes, G., *et al*. (2012). "Distinct microbial populations exist in the mucosa- associated microbiota of sub- groups of irritable bowel syndrome." Neurogastroenterology & Motility **24**(1): 31-39.

Quast, C., *et al*. "J Peplies, Glöckner FO. 2013." The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research **41**: D590-D596.

Riedl, A., *et al*. (2008). "Somatic comorbidities of irritable bowel syndrome: a systematic analysis." Journal of psychosomatic research **64**(6): 573-582.

Ríos-Covián, D., *et al*. (2016). "Intestinal short chain fatty acids and their link with diet and human health." Frontiers in microbiology **7**: 185.

Sasani, T. A., Cone, K. R., Quinlan, A. R., & Elde, N. C. (2018). Long read sequencing reveals poxvirus evolution through rapid homogenization of gene arrays. *eLife, 7*, e35453.

Spiller, R. C. (2007). Role of infection in irritable bowel syndrome. *Journal of gastroenterology, 42*(17), 41-47.

Scholz, M., *et al*. (2016). "Strain-level microbial epidemiology and population genomics from shotgun metagenomics." Nature methods **13**(5): 435-438.

Sikaroudi, M. K., *et al*. (2020). "Vitamin D3 Supplementation in Diarrhea-Predominant Irritable Bowel Syndrome Patients: The Effects on Symptoms Improvement, Serum Corticotropin-Releasing Hormone, and Interleukin-6–A Randomized Clinical Trial." Complementary Medicine Research: 1- 8

Singh, R. K., *et al*. (2017). "Influence of diet on the gut microbiome and implications for human health." Journal of translational medicine **15**(1): 73.

Sperber, A. D., *et al*. (2017). "The global prevalence of IBS in adults remains elusive due to the heterogeneity of studies: a Rome Foundation working team literature review." Gut **66**(6): 1075- 1082.

Simrén, M., *et al*. (2013). "Intestinal microbiota in functional bowel disorders: a Rome foundation report." Gut **62**(1): 159-176.

Sainsbury, A., *et al*. (2013). "Prevalence of irritable bowel syndrome–type symptoms in patients with celiac disease: a meta-analysis." Clinical Gastroenterology and Hepatology **11**(4): 359-365. e351.

Singh, V. V. and P. P. Toskes (2004). "Small bowel bacterial overgrowth: presentation, diagnosis,and treatment." Current treatment options in gastroenterology **7**(1): 19-28.

Szabo, G., *et al*. (2010). "Gut-liver axis and sensing microbes." Digestive diseases **28**(6):

737-744. Saito, Y., *et al*. (2007). "A genetic association study of 5- HTT LPR and GNβ3 C825T polymorphisms with irritable bowel syndrome." Neurogastroenterology & Motility **19**(6): 465-47 Scarpellini, E., *et al*. (2007). "Efficacy of butyrate in the treatment of diarrhoea-predominantirritable bowel syndrome." Digestive and Liver Disease Supplements **1**(1): 19-22.

Shah, A., *et al*. (2019). "Link between celiac disease and inflammatory bowel disease." Journal ofclinical gastroenterology **53**(7): 514-522.

Shah, K., *et al*. (2019). "Mind-body treatments of irritable bowel syndrome symptoms: An updatedmeta-analysis." Behaviour Research and Therapy: 103462.

Tarnowski, W., *et al*. (2011). "Outcome of treatment with butyric acid In irritable bowel syndrome–preliminary report." Gastroenterol Prakt **1**: 43-48.

Thompson, W., *et al*. (2000). "Irritable bowel syndrome in general practice: prevalence,characteristics, and referral." Gut **46**(1): 78-82.

Tran, T. Q., Cherezov, A., Du, X., Park, J., & Lee, D. (2019). *Development of hexagonal-z geometry capability in RAST-K for fast reactor analysis*.

Tana, C., *et al*. (2010). "Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome." Neurogastroenterology & Motility **22**(5): 512-e115.

Vander Heiden, M. G., *et al*. (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." science **324**(5930): 1029-1033.

Wade, W. (2002). Unculturable bacteria—the uncharacterized organisms that cause oral infections.

Journal of the Royal Society of Medicine, 95(2), 81-83.

Wang, Z., *et al*. (2019). "Characteristic dysbiosis of gut microbiota of Chinese patients with diarrhea-predominant irritable bowel syndrome by an insight into the pan-microbiome." Chinese medical journal **132**(8): 889.

Watson, A. K., *et al*. (2019). The Methodology Behind Network Thinking: Graphs to Analyze Microbial Complexity and Evolution. Evolutionary Genomics, Springer**:** 271-308.

Walker, A. W., *et al*. (2015). "16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice." Microbiome **3**(1): 26.

Wang, W., *et al*. (2003). "Wang *et al* 2003." Electrophoresis **24**: 2369-2375.

Xu, D., *et al*. (2019). "Efficacy of fecal microbiota transplantation in irritable bowel syndrome: a systematic review and meta-analysis." American Journal of Gastroenterology **114**(7): 1043-1050.

Xu, D., *et al*. (2019). "Efficacy of fecal microbiota transplantation in irritable bowel syndrome: a systematic review and meta-analysis." American Journal of Gastroenterology **114**(7): 1043-1050.

Yamaguchi, Y., *et al*. (2016). "Association of intestinal microbiota with metabolic markers anddietary habits in patients with type 2 diabetes." Digestion **94**(2): 66-72.
Yatsunenko, T., *et al*. (2012). "Human gut microbiome viewed across age and geography. Nature **486**(7402): 222-227.

Zapater, P., *et al*. (2013). "Protective effect of Bifidobacterium pseudocatenulatum CECT7765 against induced bacterial antigen."

Zeber-Lubecka, N., *et al*. (2016). "Limited prolonged effects of rifaximin treatment on irritable bowel syndrome-related differences in the fecal microbiome and metabolome." Gut Microbes **7**(5): 397-413.

Załęski, A., *et al*. (2013). "Butyric acid in irritable bowel syndrome." Przeglad gastroenterologiczny **8**(6): 350.Zhou, J., *et al*. (2008). "Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents." American Journal of Physiology- Endocrinology and Metabolism **295**(5): E1160-E1166.

Zhou, X.-Y., *et al*. (2016). "Visceral hypersensitive rats share common dysbiosis features with irritable bowel syndrome patients." World journal of gastroenterology **22**(22): 5211.

Zoetendal, E., *et al*. (2008). "High-throughput diversity and functionality analysis of thegastrointestinal tract microbiota." Gut **57**(11): 1605-1615

Table A1: Composition of TE Buffer

Solutions	Quantity per litre
1 M Tris-HCl (pH 8)	10 ml
0.5 M EDTA (pH 8)	2 ml
Distilled water	988 ml

Table A2: Composition of TBE Buffer

Solutions	Quantity per litre
1 M NaOH	80 ml
1 M Tris base (pH 8)	400 ml
1 M Boric acid	400 ml
Distilled water	120 ml

Table A3: Composition of reaction mixture

Components of reaction mixture	Quantity per 20 µL
Template DNA	4 µL
5 X master mix (Affymetrix, USB products,USA)	4 µL
Primer (Alpha DNA, Canada)	1 µL of each R and F primer
PCR water (Sigma-Aldrich, USA)	10 µL

Molecular Biology Reports (2021) 48:5371–5376
https://doi.org/10.1007/s11033-021-06540-5

SHORT COMMUNICATION

Genomic-based characterization of *Enterococcus* spp.: an emerging pathogen isolated from human gut

Zumara Younus[1,2] · Sagar M. Goyal[2] · Vikash Singh[2] · Aamer Ikram[3] · Muhammad Imran[1]

Received: 1 March 2021 / Accepted: 2 July 2021 / Published online: 7 July 2021

Abstract

Background Enterococci are ubiquitous microorganisms having diverse ecological niches but most prominently in gastrointestinal tract of humans and animals. Production of enterocins makes them a good probiotic candidate. However, their role as probiotics has become ambiguous in the last few years because of the presence of virulence factors and antibiotic resistance genes. These virulence traits are known to be transferred genetically, which makes them opportunistic pathogens in the gastrointestinal tract leading to serious concerns about their being used as probiotics. In the present study, *Enterococcus*spp. isolated from the human gut were subjected to Whole-Genome Sequencing (WGS) to determine the presence of resistance and virulence genes.

Methods and results Four human origins *Enterococcus* spp. including *Enterococcus faecalis, Enterococcus casseliflavus,* and two *Enterococcus gallinarum* were isolated from human fecal samples and further cultured on blood agar. Sanger sequencing was done using Applied Biosystems 3730xl DNA Analyzer. These strains were further subjected to WGS using oxford nanopore technology MinION. Raw data were analyzed using the free online tool epi2me. The Comprehensive Antibiotic Resistance Database (CARD) and RAST (Rapid Annotation using Subsystem Technology) software were used to look for the presence of antibiotic resistance genes in these strains. Resistance determinants for clinically important antibiotics (vancomycin) and functional virulence factor genes were detected. G-view server was used for comparative genomics of all strains.

Conclusion The genomic sequencing of *Enterococcus* suggested that *E. faecalis, E. casseliflavus*, and *E. gallinarum* strains are opportunistic pathogens, having antibiotic resistance genes. All isolates had vancomycin resistance genes, which were expressed phenotypically. Genes related to bacteriocin resistance were also present in *E. casseliflavus* and *E. gallinarum*.

Keywords *Enterococcus* spp. · Probiotics · Whole genome sequencing · Antimicrobial resistance · Vancomycin · MinION · Sanger sequencing

Introduction

Enterococcus genus is an important class of lactic acid bacteria (LAB) in the phylum Firmicutes that can survive in diverse ecological niches [1] including intestines of humans and animals and in food products. Most strains of enterococci are proven to have probiotic properties and are considered safe for hosts [2]. Probiotic bacteria are known for centuries mainly for their health benefits mostly in metabolic disorders. The Food and Agriculture Organization (FAO) and World Health Organization (WHO) have established some basic criteria before considering a bacterium as probiotic, e.g., tolerance level against gastrointestinal transit, production of antimicrobial peptides, susceptibility to antibiotics, and having immunomodulation activity [3].

Several genera of lactic acid bacteria (LAB) such as *Aerococcus, Carnobacterium* and *Enterococcus* have been studied due to their potential probiotic capability [4]. However, due to the presence sometimes of certain genes, their role is not as positive as thought to be previously. Hence, genomic analysis is useful in the identification and study of

✉ Muhammad Imran
mmimran@qau.edu.pk

[1] Department of Microbiology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad 45320, Pakistan

[2] Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota, MN 55108, USA

[3] National Institute of Health (NIH), Park Road, Islamabad 45550, Pakistan

Turnitin Originality Report

Implication of Gut Microbiome in Pathophysiology of Irritable Bowel Syndrome (IBS) by turnitin
Zumara Younus

From Review (Review)

- Processed on 10-Nov-2021 23:27 PKT
- ID: 1698958515
- Word Count: 36542

Similarity Index
19%
Similarity by Source

Internet Sources:
16%
Publications:
8%
Student Papers:
3%

sources:

1 1% match (publications)
"Oral Presentations", Neurogastroenterology & Motility, 2010

2 1% match (student papers from 06-Dec-2017)
Submitted to Higher Education Commission Pakistan on 2017-12-06

3 < 1% match (Internet from 24-May-2019)
https://link.springer.com/article/10.1007%2Fs10620-015-3932-1

4 < 1% match (Internet from 10-Jan-2020)
https://link.springer.com/article/10.1007%2Fs10096-017-3060-2

5 < 1% match (Internet from 24-Sep-2019)
https://link.springer.com/article/10.1007/s12325-018-0673-5

6 < 1% match (Internet from 08-Jul-2019)
https://link.springer.com/article/10.1007%2Fs11894-013-0323-7

7 < 1% match (Internet from 14-Jan-2019)
https://link.springer.com/article/10.1007/s10620-015-3607-y

8 < 1% match (Internet from 24-Apr-2019)
https://link.springer.com/content/pdf/10.1007%2F978-3-319-56360-2.pdf

9 < 1% match (Internet from 05-Nov-2017)
https://link.springer.com/content/pdf/10.1007%2F978-3-319-06151-1.pdf

10 < 1% match (Internet from 27-Oct-2021)
https://link.springer.com/article/10.1007/s10620-015-3607-y?code=4eb54814-16b4-4c20-8a6b-8f76654ef065&error=cookies_not_supported

11 < 1% match (Internet from 13-Nov-2017)
https://link.springer.com/content/pdf/10.1007%2Fs11894-017-0554-0.pdf

12 < 1% match (Internet from 04-Aug-2019)
https://link.springer.com/article/10.1007%2Fs00436-018-6021-5

13 < 1% match (Internet from 26-Nov-2019)
https://link.springer.com/article/10.1007%2Fs00702-019-02083-z

14 < 1% match (Internet from 07-Nov-2017)
https://link.springer.com/content/pdf/10.1007%2F978-1-4939-0897-4.pdf