Isolation and Characterization of Mucosa Associated Microbiota (MAM) of Stomach in Functional Dyspepsia Patients Among Pakistani Population

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Master of Science in Healthcare Biotechnology

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DEDICATION

To my beloved parents

for their endless love, support and encouragement

To my little brother

for being my confidant & best friend

ACKNOWLEGMENTS

"All the praises and thanks to Allah, the Lord of the heavens and the Lord of the earth, Lord of all Worlds (45:36)."

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LIST OF ACRONYMS

FD	Functional dyspepsia
MAM	Mucosa Associated Microbiota
PDS	Postprandial Distress Syndrome
EPS	Epigastric Pain Syndrome
NSAID	Nonsteroidal Anti-inflammatory Drugs
GI	Gastrointestinal
SCFA	Short Chain Fatty Acids
WGS	Whole Genome Sequencing
HMP	Human Microbiome Project
NIH	National Institute of Health Sciences
MetaHIT	Metagenomics of the Human Intestinal Tract
FGID	Functional Gastrointestinal Disorders
MBP	Major Basic Protein
EPO	Eosinophil Peroxidase

ABSTRACT

Modern advancements in the sequencing technology for analyzing microbial communities have provided valuable insights on the intricate microbiota present in mammalian gastrointestinal tract whose composition have a significant impact in determining the overall health of the host. Functional dyspepsia (FD) is a prevalent chronic functional condition affecting the gastrointestinal system. Due to scarcity of the information of its pathogenesis, the treatment of functional dyspepsia poses challenges, and for the vast majority of patients, the problem persists chronically with symptoms that vary over time. Current study was designed for the characterization of mucosa associated microbiota (MAM) of stomach among functional dyspepsia patients. Biopsy samples of patients presenting symptoms of dyspepsia were collected from Holy Family Hospital Rawalpindi. Samples were processed and cultured repeatedly to isolate pure bacterial colonies. The pure isolates were then subjected to colony PCR for the amplification of 16srRNA gene using universal primers 27F and 1492R. For the identification of the isolates, Sanger Sequencing was done with 518F and 800R primers which cover the V4 region of the 16srRNA gene. Sequencing and Phylogenetic Analysis revealed that 41% of the isolates identified belong to phyla *Firmicutes* and 59% to *Actinobacteria*. Among Firmicutes most of the isolates belong to *Lactobacillus*, while among *Actinobacteria most of isolates* belongs to the family *Micrococcaceae*. *The* phylum *Actinobacteria* was found to be highly abundant in the analyzed samples. Thus, our results indicate high prevalence of *Firmicutes* and *Actinobacteria* in stomach microbiota of patients suffering from Functional Dyspepsia in Pakistan. Overall, the current study provides a better understanding of stomach microflora associated with functional dyspepsia. Exploration of the intricate relationship between the

host and microbiota holds the potential to uncover innovative therapeutic strategies that specifically and effectively cure different aspects of the disease.

INTRODUCTION

Functional dyspepsia is the most prevalent gastroduodenal disorder which causes recurring symptoms like epigastric pain, burning sensation, early satiety, nausea, postprandial fullness (Zhou et al., 2022). Despite being chronic, it causes intermittent gastrointestinal symptoms which have no organic causes (Zheng et al., 2022). But it is known to cause alteration in the normal gastrointestinal microflora. ROME IV criteria have classified functional dyspepsia into two categories, Postprandial Distress Syndrome (PDS) and Epigastric Pain Syndrome (EPS) and PDS-EPS overlaps (Stanghellini et al., 2016).

Almost 80% of the patients having dyspepsia have no structural cause of the disease like abnormal gastrointestinal refluxes, peptic ulcers etc. and have functional dyspepsia. Only 20-30% of the patients having symptoms visit hospitals. Functional Dyspepsia (FD) is prevalent in almost 16% of the general population and 80% of the population (Ford et al., 2020). Few studies show a rate of 61% for postprandial distress syndrome (PDS), 18% of epigastric pain syndrome (EPS) and 21% of the symptoms of population overlapped with both syndromes (Aziz et al., 2018).

Several epidemiological investigation indicates that female sex, smoking, the administration of nonsteroidal anti-inflammatory drugs (NSAID), and *H. pylori* infection are factors contributing to dyspepsia in general population (Ford et al., 2020). FD have been found to have an adverse impact on both the patient's quality of life and productivity. It has become one of the primary medical concerns of the modern era and has garnered

widespread concern because of the economic burden on patients as well as the healthcare system (Zheng et al., 2022).

Functional dysbiosis pathogenesis has not yet been completely understood, it is predominantly considered to be associated with different pathophysiological factors which include alteration in gastrointestinal microbiota, genetic predisposition, gastric hypersensitivity, abnormal motility of duedojejunal, CNS dysfunction or *H. pylori* infection (Kumar et al., 2022).

100 trillions of commensal bacteria reside in the gut microbiome and are crucial for the maintenance of various physiological processes including the functioning of gastrointestinal barrier, as well as immunological and metabolic processes. Lately, there has been a surge towards investigating the association between gastrointestinal tract and human health and disease. Several studies have documented that the onset and occurrence of various human diseases is associated with gastrointestinal dysbiosis (Zhou et al., 2022).

Due to the extreme acidic (pH<4) conditions of the stomach, it was considered that no bacteria could colonize there. But recent studies revealed many bacteria have adapted to these conditions which mostly includes obligate or facultative acidophiles. Several investigations have shown evidence for the presence of a unique microbiota associated with the stomach, in addition to *H. pylori*. In microbiota of a healthy person, the phyla Firmicutes and Proteobacteria exhibit the highest abundance level in gastric mucosal samples, whereas gastric fluid samples are primarily characterised by the prevalence of Firmicutes, Bacteroidetes, and Actinobacteria (Wu et al., 2014). Whereas several studies report abundance in *Streptococcus, Actinomyces, Proteobacteria, Firmicutes,* and *Bacteroidetes* among functional dyspepsia patients. Still, we know very little about the

dysbiosis of gastrointestinal microbiota and how it is involved in causing Functional dyspepsia (FD). Evidently, the perturbation in the quantity and diversity of the microbiome of gastrointestinal (GI) tract has a substantial role in the development and advancement of functional dyspepsia. Also, insufficient data exists pertaining to the prevalence of dyspepsia in Pakistan. Therefore, this study is designed to find the association of the stomach microbiota which leads to the pathogenesis of functional dyspepsia (FD) among Pakistani population which will assist in better understanding of gastrointestinal microbiota.

LITERATURE REVIEW

2.1 Gut Microbiota

2.1.1 The Microbiome

The microbiome of gastrointestinal (GI) tract plays a significant role in host development which involves an extensive spectrum of physiological processes and disease pathogenesis like regulation of GI development and immune system, metabolism of complex compounds and maintenance of body homeostasis (Hansen et al., 2020). Previously, the stomach microbiota was considered to be a hostile environment for colonization and survival of bacteria due to its harsh and acidic nature and paucity of molecular identification techniques to characterize uncultured microbiota (Wu et al., 2014). But the discovery of *H. pylori* in the 1980's led us to believe that stomach is not a sterile organ (Warren et al., 1983). With the advancement in molecular techniques, it was discovered that apart from *H. pylori* there is a wide diversity of microbiota present which is known as gastric microbiota. Microbiome of the human gastrointestinal (GI) tract has an intricate ecosystem which is a home to many diverse microorganisms such as Bacteria, Archaea, viruses, and Eukarya (Gouba et al., 2019).

These microorganism all live in harmony and provide a dynamic to maintain the working of gut ecosystem. Gut microbiome is involved in retaining various physiological processes like conversion of roughage into short chain fatty acids (SCFA), regulation of gut-brain axis, synthesizing essential vitamins, and metabolites which is responsible for regulating the immune system and intestinal angiogenesis. This indicates that gut microbiota has a

mutualistic relationship with the human host rather than a commensal one (Huang et al., 2019).

Bacteria is the most predominant microbe residing in GI microbiome which includes Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Huang et al., 2019). The most prevalent phylum from bacteria are Firmicutes and Bacteroidetes although low quantity of Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria is also present. (Eckburg et al., 2005). The paradigm of microbiota is distinctly specific for each individual and differ from each other based on different factors which includes diet, environmental factors, genetic conditions, age, drugs and predisposition to various infectious diseases (Dieterich et al., 2018).

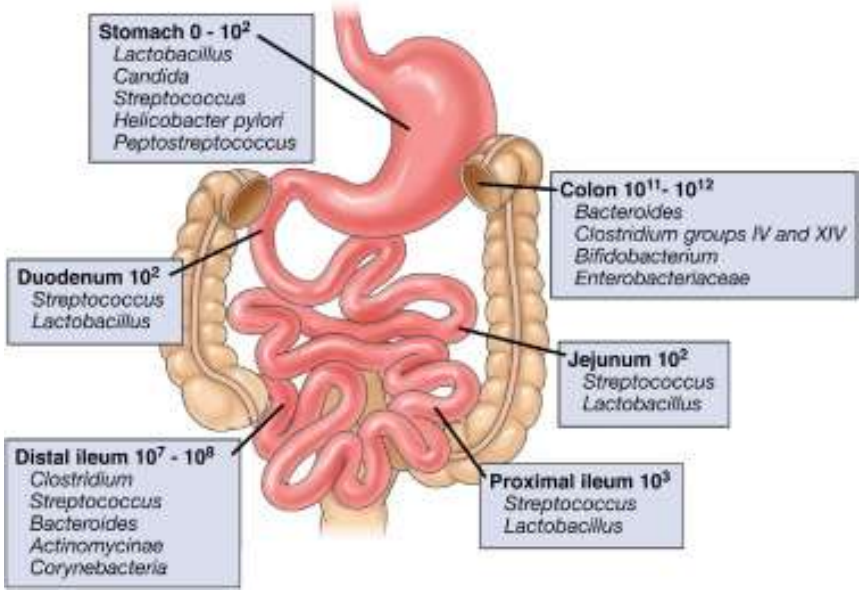

Figure 2. 1 *Distribution of dominant microbial species in different parts of the gastrointestinal tract. (Sartor et al., 2008)*

There are more than 10^{14} microorganisms per deciliter that reside in the human GI tract which constitute more genomic content than the human genome (Thursby & Juge, 2017).

Stomach is a part of this GI tract which only contain 10^1/g bacterial load because of its inhospitable environment while other parts of GI tract like duodenum constitute 10^3/g, jejunum 10^4/g, ileum 10^7/g and colon 10^{12}/g bacterial load respectively (Dieterich et al., 2018).

2.1.2 Metagenomics

The research on human gut microbiota is still at very initial stages and an extensive knowledge is required about host-microbiome relationship and how it is involved in pathogenesis of diseases and essential processes (Kho et al., 2018). Due to the advancement in molecular based techniques, identification of the microbiota present in GI has become a lot easier. 16S ribosomal RNA (rRNA) sequencing and whole genome sequencing (WGS) are widely used to distinguish microbial communities. Bacterial species have both variable and conserved regions on the 16srRNA gene which ranges from V1-V9 from which some of the sequence is conserved throughout bacterial species that is used to target in sequencing techniques and serves as a perfect reference for metagenomics analysis of microbiome (Huang et al., 2019). Broad range metagenomics projects such as Human Microbiome Project (HMP) Consortium which was capitalized by National Institute of Health Sciences (NIH) USA and MetaHIT (Metagenomics of the Human Intestinal Tract) projects capitalized by European Commission has contributed to characterize and identify the human genome through which we can broaden our research to further understand the correlation between various microbial communities and how they can impact our health (The Human Microbiome Project Consortium, 2012) (Kho et al., 2018).

2.2 Functional Dyspepsia

2.2.1 Definition

Various factors are involved in disrupting the natural microflora of intestine which causes imbalance known as dysbiosis (Isaac et al., 2019). A concise and easy definition of dyspepsia is upper abdominal pain and distress. When the symptom of dyspepsia persists for over a course of month it is considered chronic. When there is no structural or organic cause of dysbiosis it is classified as functional dyspepsia (FD) (Agréus et al., 2002). Dysbiosis of the gut microbiota causes various dysregulation which stimulate the immune system and causes different gastrointestinal diseases including FD.

2.2.2 Types of Functional Dyspepsia

Dyspeptic patients can be categorized into two main types depending upon their etiology.

- Secondary Dyspepsia which includes patients who have structural symptoms which can be identified with conventional diagnostic tools like endoscopy and the conditions ameliorates or wears off according to the disease e.g., ulcers or gastritis. Another subcategory of dyspepsia is *H. pylori* associated dyspepsia.
- Patients who have no organic or structural cause for their symptoms are simply categorized under the term functional dyspepsia (Stanghellini et al., 2016).

ROME IV criteria has divided functional dyspepsia into two distinct types of epigastric pain syndrome (EPS) and postprandial distress syndrome (PDS) and EPS-PDS overlap. (Futagami et al., 2011).

Epigastric Pain Syndrome (EPS)

Epigastric pain syndrome as the name suggests is characterized by epigastric pain or burning sensation which has been recurring for 3 months with a symptomatic history of 6 months. In addition, symptoms may include bloating, belching and nausea. Intake of food can also cause or alleviate pain (Stanghellini et al., 2016). EPS is observed in 28% of the patients having FD (Harer & Hasler, 2020).

Postprandial Distress Syndrome (PDS)

In PDS, patients suffer from early satiety and feeling of fullness which is serious enough to affect day to day tasks. Burning sensation, nausea, belching, epigastric bloating are common symptoms which occur during PDS (Stanghellini et al., 2016). 38% of the patients diagnosed with functional dyspepsia have PDS (Harer & Hasler, 2020).

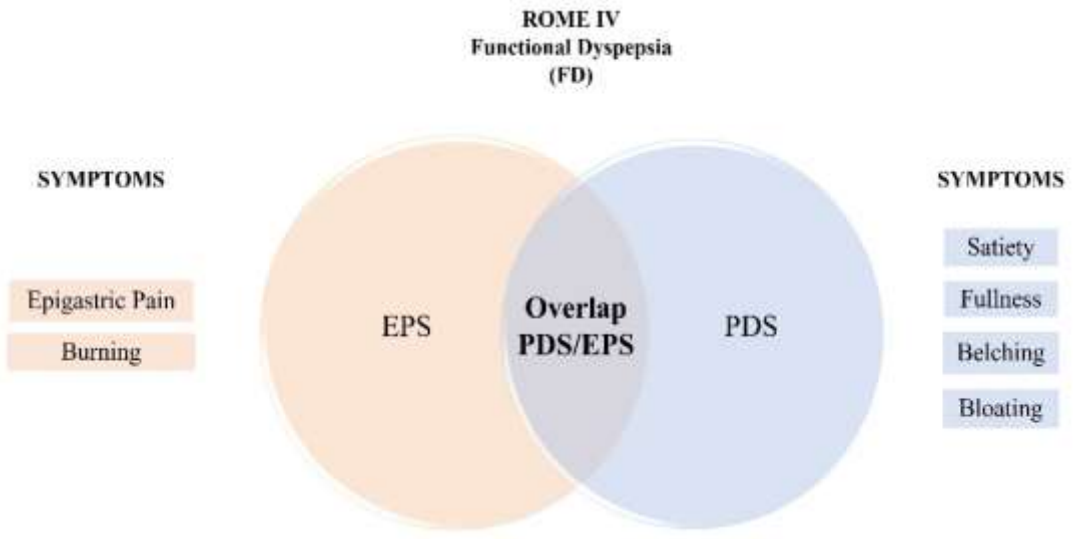

Figure 2. 2 *Rome IV criteria and classification of FD*

2.3 Pathophysiology

The pathophysiology of functional dyspepsia is very complex because of the heterogenous symptoms of the disease. To this day more research needs to be done on the gut microbiota to fully understand the complex nature of this disease.

2.3.1 Gastric Neuromuscular Dysfunction

Mainly delayed gastric emptying, failure in the relaxation of gastric fundus, postprandial distress, and visceral hypersensitivity are said to be included in gastric neuromuscular dysfunction and common in functional dyspepsia patients. Disturbance in the gastric and duodenal motility is one of the major cause of functional dyspepsia .

Gastric Emptying

Symptoms of functional dyspepsia can be justified by delayed gastric emptying which is also considered to play an important role in gastroparesis. But researchers are still not sure whether dyspepsia and gastroparesis are two individual diseases or the result of imbalance in gastric function with the same pathophysiological mechanism (Harer et al., 2020). According to different studies 20%-50% of the dyspeptic symptoms show delayed gastric emptying (Tack et al., 2004a). It is mostly seen in patients who have a feeling of heaviness and fullness after having a meal. But the symptoms are at variance with rate of gastric emptying (Ford et al., 2020). A meta-analysis reported improvement in symptoms when the gastric emptying was accelerated by 20min only when optimal method tests were used (Vijayvargiya et al., 2019). In about 2% of the FD patients rapid gastric emptying is also reported which is very rare. Patients having delayed emptying also presents with more nausea, vomiting and loss of appetite (Stanghellini et al., 2016).

Impaired Gastric Accommodation

While the distal part of the stomach is involved in gastric emptying of solids by grinding the food, the proximal part is playing an essential role in the retention of food. Relaxation of the proximal part of the stomach allows the food to retain in the stomach (Tack et al., 2004a). Vago-vagal refluxes support the retention of food in the stomach which is triggered by nitrergic nerves present in the walls of stomach (Stanghellini et al., 2016). Disruption of the motor function of proximal part can impair gastric accommodation of food and is related to early satiety in functional dyspepsia patients. When there is a problem in retaining the food in the proximal part it is pushed back into distal part which causes the antrum to be distended (Tack et al., 2004). Impaired gastric accommodation is seen in one-third of the patients suffering from functional dyspepsia (Stanghellini et al., 2016).

Visceral Hypersensitivity

Several factors have been recognized as the cause of gastric hypersensitivity. Various studies have demonstrated that sensory dysfunction is also a common symptom in FD which makes the stomach more susceptible to gastric detention. Postprandial epigastric discomfort is a usual complain among majority of the patients which indicates that cause of epigastric pain is an increased susceptibility to mechanical stress cause by gastric distension (Yarandi & Christie, 2013). Patients with organic reasons of dyspepsia are just as likely to experience upper abdominal pain, distress, and nausea (Thumshirn, 2002). Hypersensitivity is also reported to be associated with increased gastric acid secretions in the duodenum which is the cause of nausea. A study concluded that meal related symptoms like nausea, satiety was because of postprandial sensitivity to gastric detention (Tack et al., 2004). Constant exposure to capsaicin triggers Trp1 which causes the discharge of

neuropeptides that are responsible for heightened visceral sensitivity (Ford et al., 2020). In addition to this, inactive visceral nociceptors can be activated by central hypersensitivity through causing dysregulation to the pain pathway. Abnormal brain activity in FD patients also indicates a CNS impact (Yarandi & Christie, 2013). Hypersensitivity of dorsal neurons or dysfunctional supraspinal function may be contributing factors in elevated viscerosomatic pain in FD patients (Thumshirn, 2002).

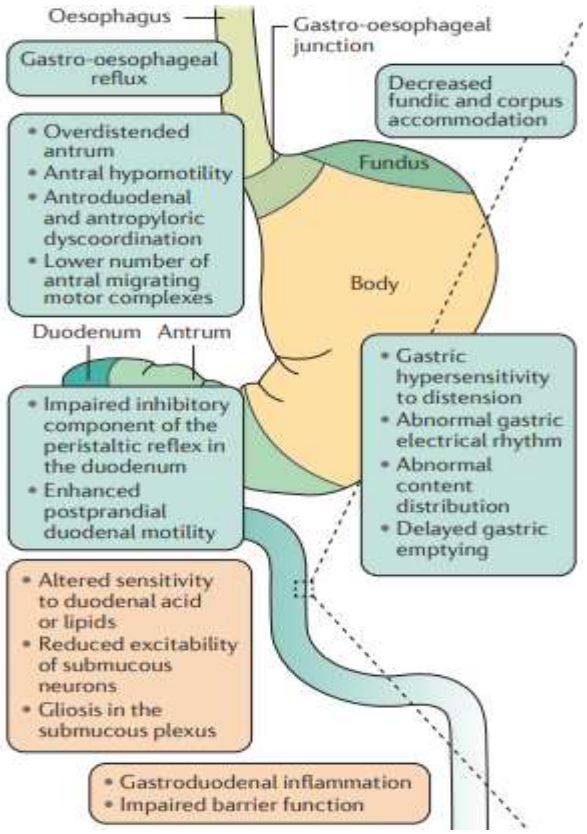

Figure 2.3 *Symptoms of Functional Dyspepsia affecting stomach function (Enck et al., 2017)*

2.3.2 Immune Dysfunction

It is known that dietary factors also play an crucial role in aggravating the symptoms of functional gastrointestinal disorders (FGID). Functional dyspepsia patients often report onset of symptoms after ingestion of food. Also, to relieve the symptoms in FD patients often dietary changes are frequently made with varying levels of success to get an enhanced grasp at immune reaction towards food which could increase the efficacy of this intervention technique.

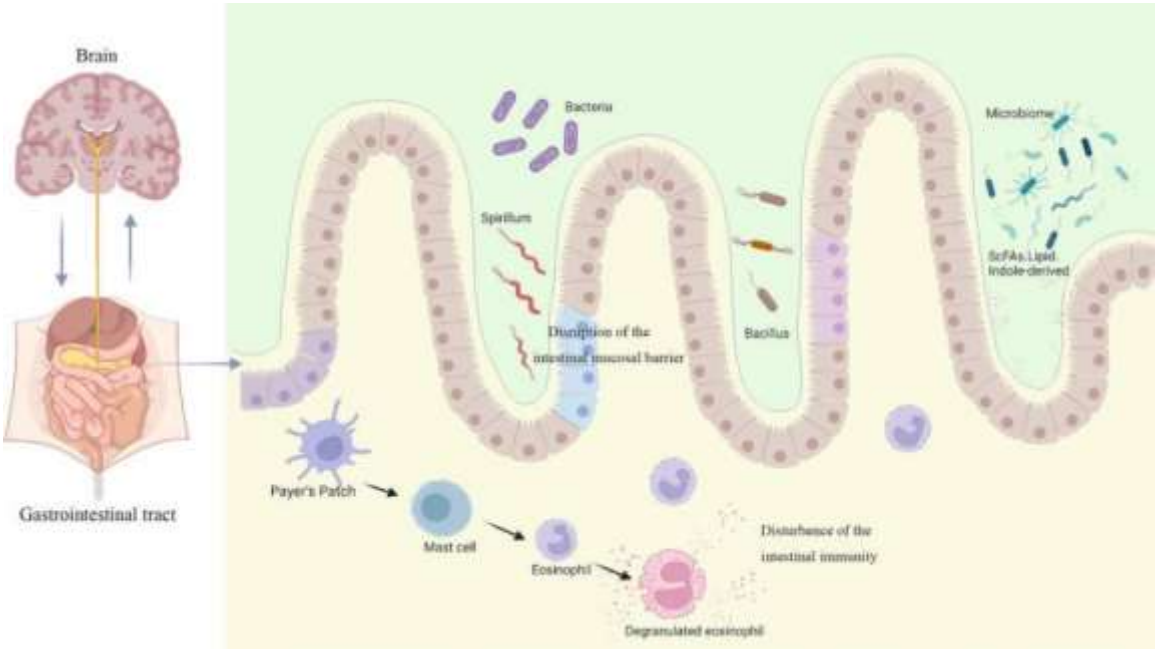

Figure 2. 4 *Pathogenesis of functional dyspepsia in correlation with gastric microbiota*

A systematic review reported an elevation in duodenal eosinophils in a group of functional dyspepsia patients. The underlying cause of this increase is not entirely comprehended specifically because of the lack of significant evidence for a Th2 response. However, degranulation of eosinophils is seen (Burns et al., 2019). Degranulation of eosinophils has a cytotoxic effect leading to the release of major basic protein (MBP) and eosinophil

peroxidase (EPO), which is in turn responsible for causing dysfunction in epithelial barrier. EPO and MBP triggers vagal receptors impairment which causes smooth muscle hyper-reactivity may exacerbate FD symptoms characterized by visceral hypersensitivity (Pryor et al., 2020).

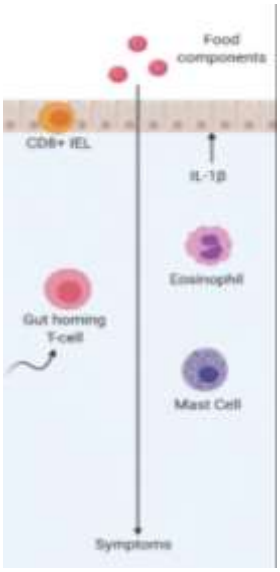

Figure 2. 5 *Pathology of Food hypersensitivity caused by immune dysfunction in functional dyspepsia. Increased production of eosinophils and mast cells is associated with the disease and causes imbalance in mucosal barrier function.*

FAS, recognized for its role in cell apoptosis and lymphocyte homeostasis, and HLA-DRA, which is responsible for the proliferation of B cells, has shown significant downregulation in FD patients which can imply changes in population of duodenal lymphocytes. Enhanced immune cell response and duodenal sensitivity may attribute to delay in gastric emptying. Impairment of neuroimmune response can also lead to disturbance in motility and hypersensitivity. (Ford et al., 2020).

2.3.2 *H. pylori* Infection

H. pylori can also cause dyspepsia like symptoms. Several studies have been executed to examine the correlation between *H. pylori* infection and dyspepsia (Thumshirn, 2002). Persistent *H. pylori* infection have the potential to be responsible for accelerated gastric emptying (Yarandi et al., 2013). Studies have reported that *H. pylori* patients with FD have dysfunction in gastric sensitivity and motility due to inflammation in muscular layer of stomach (Suzuki et al., 2013). A meta-analysis showed that after using eradication therapy for *H. pylori* symptoms deliberately improved in patients who were declared *H. pylori* negative as compared to those who remained positive (Bazzoli et al., 2002). The correlation between *H. pylori* infection with dyspeptic symptoms is still a bit of a controversy and the connection between them remains unclear and more research needs to be done to find association between the two (Lee et al., 2004).

2.3.3 Psychosocial Factors

Pathogenesis of functional dyspepsia is also widely associated with psychosocial factors. Anxiety is also a frequent symptoms, but it is not associated with depression in FD patients. A study conducted in people not having dyspepsia showed that increased anxiety can cause FD later in life suggesting presence of relationship between gut and psyche (Yarandi et al., 2013). Postprandial symptoms can proliferate remarkably with mental stress which can cause elevated level of ACTH and cortisol (De Giorgi et al., 2013). Many physicians believe that FD patients are very susceptible to developing psychological disorders and recently multiple studies providing experimental data regarding psychological factors effect on gastrointestinal function have drawn attention (Miwa et al., 2011).

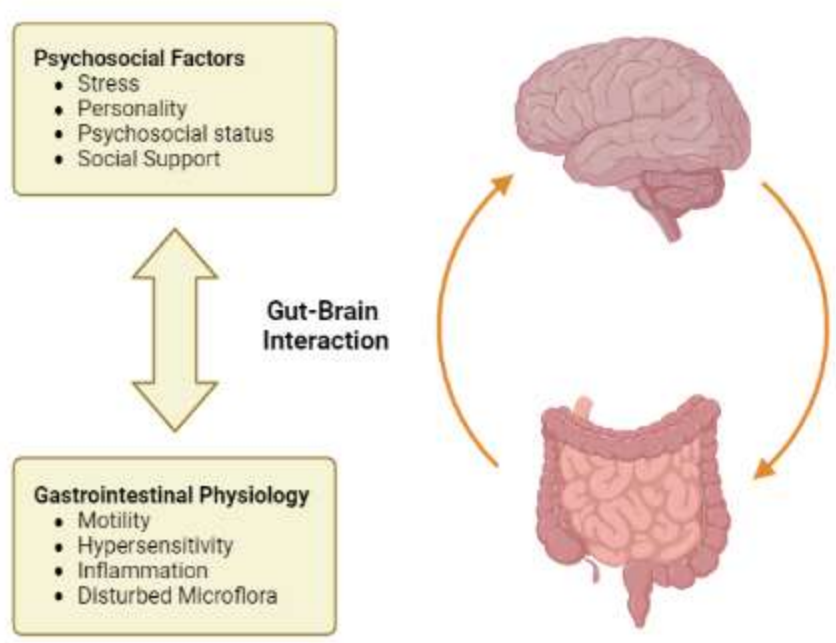

Figure 2. 6 *Representation of gut-brain interaction and effect of psychosocial factors on physiology of gastrointestinal tract which leads to progression of functional dyspepsia.*

2.3.4 Other Factors

Various additional factors like environment, diet and lifestyle have been implicated in FD. Melatonin is also reported to be involved in pathophysiology of FD (Chojnacki, 2011). In 10-20% of patients, acute infection can cause upper gastrointestinal symptoms. Post infection upper gastrointestinal syndromes have higher probability of developing infected individuals (Stanghellini et al., 2016). Stress is also a contributing factor in FD. FD patients who have a history of physical or sexual abuse were seen to have an increased gastric hypersensitivity than in patients with normal FD and was also related with altered gastric accommodation .

Dietary factors contain variables explicitly related to food consumption, calorie intake, and intolerance to particular food as well as psychological issues (Miwa et al., 2011). Symptoms of functional dyspepsia are aggravated by consumption of fatty meals, and it is

commonly associated with postprandial fullness. A study reported that infusion of duodenal lips can increase susceptibility of FD patients to gastric distention which may cause bloating and nausea (Lee et al., 2004).

Different studies have also shown that patients suffering from functional dyspepsia have an irregular lifestyle, although a clear connection between the two is not known yet. Factors influencing lifestyle could be poor socio-economic status, caffeine intake, excessive smoking, poor residential atmosphere, and long-term illness (Miwa et al., 2011).

2.4 Functional Dyspepsia and Gut Microbiota

When there is an imbalance in the gut microbiome, a series of pathological changes happen as a consequence of dysbiosis in the gastrointestinal microbiota (Zhou et al., 2022). Proteobacteria, a gram-negative bacteria, is the most widespread phyla in the human gastrointestinal tract. A diagnostic marker for finding out dyspepsia is an increased abundance in Proteobacteria population (Rizzatti et al., 2017). Firmicutes contain the majority of gram-positive bacteria. It can be classified into three groups as facultative or aerobic (*Bacillus*), anaerobic (*Clostridium*), and non-cell wall *Hymenomycetes*. Another gram-positive bacteria phylum is Actinobacteria which has a greater percentage of GC content than *Firmicutes*. *Bacteroidetes*, *Flavobacteria* and *Sphingobacillaceae* take up more than 50% of the total gastrointestinal microbiota. These are considered as the ‘four phyla’ of the GI tract. Any dysfunction or imbalance in the relative composition of these bacteria can lead to dysbiosis of microbiota (Zhou et al., 2022).

Higher population of *Bifidobacterium* and *Clostridium* and low prevalence of Prevotella was reported in FD patients by using 16sDNA for bacterial identification which have a negative correlation with PDS symptoms (Nakae et al., 2016). A study conducted to identify microbiota in the small intestine through 16rRNA identification techniques reported low level of *Actinomycete*, *Atopobium* Collin, *Leptotrichia Trevisan*, *Prevotella*, and *Veillonella* in functional dyspepsia patients in comparison with healthy patients (Zhong et al., 2017). Another study targeting the V3-V4 region of 16srRNA for identification suggest an increase in Firmicutes and Streptococcus in patients suffering from FD (Fukui et al., 2020). The impact of changes in the composition, variety, and abundance of gut microbiota on the onset and progression of functional dyspepsia (FD) is evident (Zhou et al., 2022). The table below summarizes studies conducted to characterize stomach microbiota.

References	**FD/Controls**	**Technique for Microbiota Identification**	**Principle Findings**
Mucosa Associated Microbiota			
Zhong et al. 2017	9/9	16S rRNA gene sequencing	High abundance of *Streptococcus* and Low prevalence of *Actinomycetes* and *Prevotella*, while the overall relative abundance of bacteria exhibited a positive correlation with the intensity of clinical symptoms.
Fukui et al. 2020	11/7	16S rRNA V3-V4 gene sequencing	High prevalence of *Firmicutes* and *Streptococcus*. There was a positive correlation seen between the overall prevalence of *Streptococcus* and the occurrence of upper gastrointestinal symptoms.
Sterbiniet al. 2016	24	16S rRNA gene pyrosequencing	High Prevalence of *Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria*;
Shanahan et al. 2018	26/10	16S rRNA gene sequencing	Prevalence of *Veillonella*
Gastric Fluid Aspirate			
Nakae et al. 2016	44/44	16S rDNA gene sequencing	High levels of *Bifidobacterium* and *Clostridium, Prevotella*. Inverse relation between Prevotella and symptoms of PDS-FD
Igarashi et al. 2017	21/21	16S rRNA gene sequencing	Higher abundance of *Bacteroidetes* and absence of *Acidobacteria*

Table 2. 1 *Analysis of microbiota among Functional Dyspepsia Patients*

2.5 Epidemiology

Functional dyspepsia have been found to have an adverse impact on the quality of life and imposes a substantial socioeconomic burden on medical and healthcare facilities. The overall incidence rate of dyspepsia documented differs substantially because of the different definitions used in studies. According to large demographic studies prevalence of functional dyspepsia ranges from 10% to 30% of adults and 3.5-27% in children worldwide (Mahadeva, 2006) (Drago et al., 2021). A global study of worldwide prevalence of gastrointestinal diseases showed prevalence rate as functional dyspepsia 4.8% , PDS 3.5%, EPS 1.9%, belching disorder 0.7% (Sperber et al., 2021). Domestic International Gastro Enterology Surveillance Study (DIGEST) conducted a survey which reported that one third of the normal person have dyspepsia symptoms which included 6.5% acute dyspepsia patients and 22.5% chronic dyspepsia patients (Madisch et al., 2018a).

The incidence rate of uninvestigated dyspepsia (UD) and functional dyspepsia (FD) is 8-30% and 8-23% (Ghoshal et al., 2011). An epidemiology of functional gastrointestinal disorders was conducted in Karachi, Pakistan which reported that functional dyspepsia was the most prevalent with an incidence rate of 70.2% characterized by heartburn and bloating (Abid et al., 2022).

2.6 Treatment of Functional Dyspepsia (FD)

Upon confirmation of functional dyspepsia, an initial treatment approach involves providing a comprehensive overview of the diagnosis and its associated implications to the patient. For successful outcome of the treatment, it is very important to address the

diagnosis to patients in a very clear and concise manner emphasizing functional dyspepsia can manifest as a result of diverse underlying conditions. Simultaneously, it is imperative to provide the patient with comprehensive information regarding the available therapy alternatives (Madisch et al., 2018b).

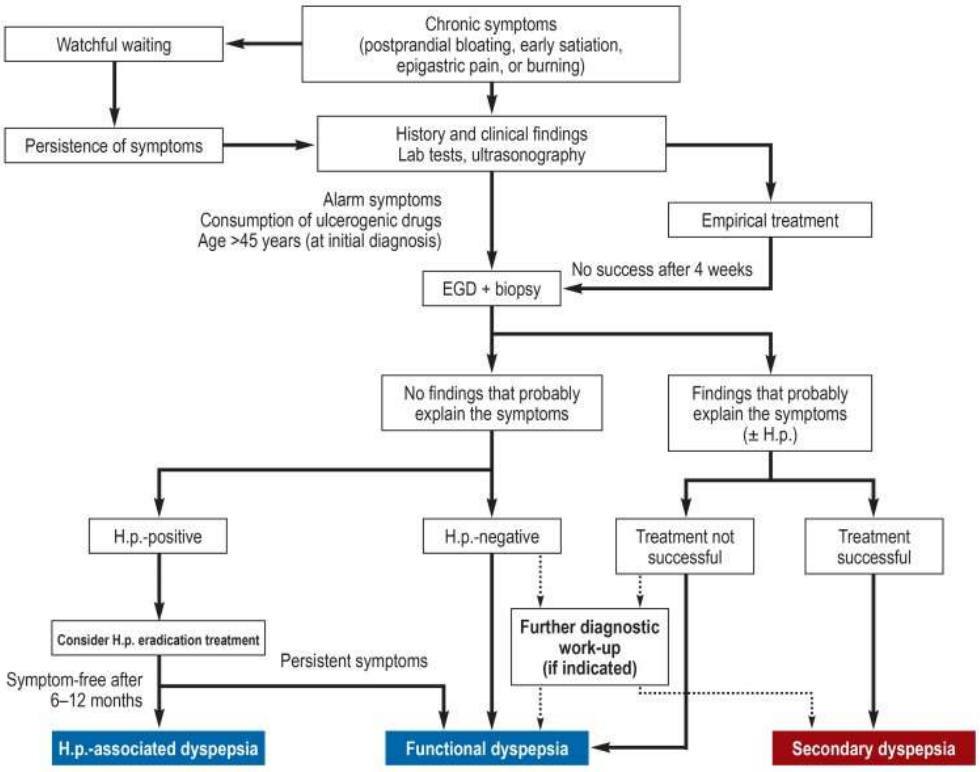

Figure 2.7 The diagnostic approach employed in individuals presenting with dyspeptic symptoms (Madisch et al., 2018).

The diagnostic approach often uncovers endoscopic and histological abnormalities that are commonly associated with gastritis. Patients diagnosed with functional dyspepsia may receive the diagnosis of "gastritis" based on the findings from endoscopic examinations and histological analyses. But “gastritis” termed should not be used as a clinical diagnosis and instead opt for "functional dyspepsia," mostly due to the lack of correspondence between the endoscopic and histological findings of gastritis and the symptoms experienced by patients (Enck et al., 2017).

Medicinal treatment is predominantly advised as a supportive intervention during the periods characterised by symptoms (Moayyedi et al., 2017). There exists a range of evidence-based therapy options, both pharmaceutical and nonmedicinal, that can be categorized as follows:

- Proton pump inhibitors
- Helicobacter pylori eradication treatment
- Phytotherapy
- Antidepressants
- Psychotherapy
- Probiotics

Acid suppressing agents and *H. pylori* eradication therapies have exhibited a notable beneficial impact on the management of functional dyspepsia when compared to the placebo. A meta-analysis revealed that the treatment effect of PPI was 10 to 20% greater compared to placebo. it has been shown that the effects of proton pump inhibitors (PPIs) are specifically confined to the treatment of epigastric pain syndrome or dyspeptic symptoms that are accompanied by reflux (Wang et al., 2007). PPIs replaced H_2 antagonistic as the initial therapeutic approach for the management of gastritis related diseases. The extensive utilization of proton pump inhibitors (PPIs) has led to the emergence of new findings regarding long-term side effects that were previously undocumented. These include an elevated likelihood of developing kidney, liver, and cardiovascular diseases, dementia, enteroendocrine tumors in the gastrointestinal tract, heightened vulnerability to respiratory and gastrointestinal infections, and compromised nutrient absorption (Yibirin et al., 2021).

Phytotherapy have also been known to treat functional dyspepsia by using combined therapeutic options. Generally, these formulations are composed of specified proportions of peppermint and caraway oil, or blends of bitter candytuft, wormwood, gentian, and angelica root, typically supplemented with spasmolytic and calming extracts such as chamomile, peppermint, caraway, and lemon balm. Phototherapeutics have been observed to elicit a sedative impact on the gastrointestinal tract, perhaps offering relief from the symptoms associated with functional dyspepsia.

Antidepressants are prescribed subsequent to the lack of success of the aforementioned interventions. Research has also indicated that antidepressants have demonstrated notable efficacy in addressing symptoms of dyspepsia, particularly when the primary complaints involve gastrointestinal distress and/or concurrent mental health issues (Madisch et al., 2018).

Probiotics have also been widely used to treat the imbalance caused by functional dyspepsia in gastrointestinal tract. Numerous research has substantiated the efficacy of probiotics in regulating the gastrointestinal microbiota, establishing their safety and effectiveness in the management of functional dyspepsia (FD). This finding implies an effective approach for the clinical management of Functional Dyspepsia (FD).

The diverse array of treatments available is indicative of the prevailing uncertainty about the underlying causes of the condition and the absence of effective therapeutic options. All these therapies have an adverse effect on the distribution of microbiota in the stomach, if not administered properly, which could further lead to the progression of functional dyspepsia. The composition of the gut microbiota in the human body is subject to various influences, such as dietary patterns, lifestyle choices, medication usage, genetic

predispositions, environmental factors, and stressors. Various variables exert an influence on the organisations and diversity of the gut microbiome. The study of relationships between host disorders and the gastrointestinal flora is currently tough for humans because of the individual heterogeneity of the gastrointestinal microbiota, which poses difficulties in terms of approaches and tools (Zhou et al., 2022). So, there is an essential need to characterize microbiota of stomach of Functional Dyspepsia (FD) patients so personalized therapeutic strategies could be devised.

METHODOLOGY

3.1 Sample collection

This research was conducted to investigate the alteration caused by functional dyspepsia in the normal microbiota of stomach. For this purpose, samples of patients suffering from gastric diseases were collected. The study was done in Atta-Ur-Rehman School of Applied Biosciences (ASAB), NUST in collaboration with Centre of Liver and Digestive Diseases of Holy Family Hospital, Rawalpindi. Prior to conducting the study, approval was received from both organizations.

3.1.1 Selection Criteria

Biopsy samples of corpus and antrum of the stomach were collected from functional dyspeptic patients who were selected on the basis of ROME IV criteria from Centre for Liver and Digestive Diseases, Holy Family Hospital Rawalpindi.

3.1.2 Inclusion Criteria

Age Range: 25-58

Fits ROME IV criteria.

3.1.3 Exclusion Criteria

Patients who have HCV associated dyspepsia.

The presence of any other disease that could be responsible for causing gastritis.

Patient who didn't give consent.

3.1.4 Sampling Procedure

Consent was taken from all the patients who met the inclusion criteria before taking the samples. Those patients whose samples were available, but they didn't give consent, were excluded. Clinical history and general information of the patients was also acquired. Consent forms are attached in the appendices.

Collection of Sample

Antrum and corpus samples were collected from the stomach by the gastroenterologist on duty and shifted in collection tubes containing 3ml PBS and were taken to Virology Lab-II ASAB, NUST.

Sample Size

A total of 28 samples were collected from the individual having functional dyspepsia symptoms.

3.2 Sample Preparation

Samples were processed within 3 hours of the sample collection to prevent bacterial diminution and contamination. Each sample was shifted in a 50ml falcon tube in a laminar flow hood to avoid contamination. again, homogenizer was first cleaned with alcohol then again with PBS so there would be no residual contamination of alcohol in the biopsy sample. Samples were homogenized at 5000rpm with a homogenizer for 30sec until the biopsy was consistent throughout PBS. Then the samples were taken for further processing.

3.3 Bacterial Enrichment

Blood agar was used for the enrichment of bacteria present in the sample. Agar was weighed accordingly, dissolved in distilled water, and then autoclaved at 121ºC for 30min. After the media was cooled down at room temperature, 7% horse blood was added in it and swirled so it would be distributed evenly. Then it was poured into petri plates and was left at room temperature to solidify in a laminar flow hood to prevent contamination. 100µl of the homogenized sample was poured onto the solid blood agar plate with the help of a sterile pipette. Then it was spread evenly on the surface of blood agar with a sterile L-shaped glass rod and labelled respectively. Petri plates were then sealed with paraffin and were put invertedly into an airtight container and incubated under microaerophilic condition with Oxoid™ CampyGen™ 2.5L Sachet at 37ºC for 72 hours.

3.4 Morphological Characterization

Isolated colonies were characterized based on their physical and morphological properties like shape, color size and hemolytic activity on the blood agar.

3.5 Bacterial Isolation

After 72 hours fresh colonies were obtained on blood agar plate which were differentiated on the basis of their morphological characteristic. 2-3 colonies were picked from each plate and then sub cultured again so pure isolated colony can be isolated through streak plate method. Blood agar plates were made again, and a single bacterial colony was picked from the reference plate with an inoculating loop which was sterilized after heating it in the

flame of Bunsen Burner. Then it was inoculated on the blood agar plate through T-shaped streak plate method. The petri plates were labelled respectively and were again incubated under microaerophilic conditions at 37ºC for 72 hours.

3.6 Colony PCR

Bacterial colony PCR was performed for the amplification of the 16SrRNA gene of the bacteria. Commercially available degenerate primer for 16srRNA gene i.e., 27F and 1492R was ordered through Macrogen Korea which covers almost full-length of 16srRNA gene. The sequence of the forward and reverse primers is as follow.

16srRNA Primers	
Forward Primer (27F)	*AGAGTTTGATCMTGGCTCAG*
Reverse Primer (1492R)	*TACGGYTACCTTGTTACGACTT*

Table 3. 1 *Universal primer 27f and 1492R for amplification of 16srRNA gene*

Figure 3. 1 *Forward and Reverse Primer of 16srRNA gene*

For the DNA extraction from the colony, 20µl of NF was taken in a PCR tube and subsequent colonies were picked carefully from the blood agar plate and was dissolved into the NF water until the solution became turbid. Tubes were spun in mini spin centrifuge. Then the tubes were placed in a boiling water bath at 95ºC for 10 minutes for the lysis of bacteria to release the DNA. After this, they were centrifuged at 6000rpm for 3 min. Pellet was discarded and supernatant was used as DNA template.

Conventional PCR was performed on a thermocycler by Applied Biosystem (model no. 2720). The amplification condition contained initial denaturation stage at 94ºC for 5 min and 30 cycles of denaturation stage for 45 seconds, annealing at 58ºC for 45 seconds, an extension at 72ºC for 45 minutes and a final extension stage at 72ºC for 8 minutes. The rection mixture composition is as follow.

Water	*20µl*
2x Fermantas PCR master mix	*25µl*
Forward Primer	*1.5µl*
Reverse Primer	*1.5µl*
DNA Template	*2µl*
Total	***50µl***

Table 3. 2 *Reaction mixture composition for the amplification of 16srRNA gene*

3.7 Quality Assessment through Gel Electrophoresis

Verification of the amplification of 16srRNA gene was assessed by gel electrophoresis. 2% TAE (Tris-acetate-EDTA buffer) agarose gel was prepared, and gel was stained by adding 7µl of 0.5 µg/ml ethidium bromide in it. 7µl of the PCR product was mixed with 3µl of the DNA loading dye and was loaded into the wells of agarose gel. 3µl of 100bp ladder by Thermo Scientific™ was run alongside with it for 45 minutes with 95 volts. Bands of the PCR product and ladder were visualized under a UV transilluminator.

3.8 Identification of Bacteria by 16SrRNA sequencing

3.8.1 Purification of PCR Product

Purification of the PCR product is an essential factor for successful sequencing because the presence of any kind of contaminant can reduce the signal intensity which can result in

unreadable sequences. Purification of the PCR product was carried out by using HighPrep™ DTR Clean-up System.

PCR product was mixed with 1.8X of binding bead solution, 81 µl of magnetic beads were used for 45 µl of PCR product. The tube was placed in a magnetic stand so that the beads can capture the PCR product. Supernatant was removed and beads were washed by adding 200µl of 80% ethanol and was mixed gently. Ethanol was removed and the washing step was repeated. After that 40µl elution buffer was added to the beads for elution purpose. Tube was placed back in the magnetic stand so that the beads can capture the elution buffer. Transfer the elute into another tube to be loaded on the sequencer.

3.8.2 Cycle Sequencing PCR

Purified PCR product was used as a template for cycle sequencing PCR. Primers covering the conserved region V4 in 16srRNA gene were ordered from Macrogen, Korea. The sequence of the forward and reverse primer is as follow.

16srRNA Sequencing Primers	
Forward Primer (518F)	*CCAGCAGCCGCGGTAATACG*
Reverse Primer (800R)	*TACCAGGGTATCTAATCC*

Table 3. 3 *Sequencing Primers for the amplification of V4 region of 16srRNA gene*

Figure 3. 2 *Forward and Reverse Primer of V4 region of16srRNA gene*

Sequencing was done with BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Cat No.4337455). The following table shows the component used.

Component	***Volume***
Big Dye	*2 µl*
Buffer	*2 µl*
Primer	*0.5 µl*
Template	*2 µl*
dH_2O	*3.5 µl*
Total Volume	***10 µl***

Table 3. 4 *Reagents used for cycle sequencing PCR.*

3.8.3 Dye Terminal Purification

HighPrep DTR kit (Cat. No DT-70005) was used to clean the sequencing product. 10µl of the sequencing product was taken and mixed with 10µl of HighPrep DTR reagent and was gently mixed with pipetting up and down. 40µl of freshly prepared 85% ethanol was added and mixed it well by pipetting up and down. Mixture was incubated at room temperature for 5 min. Tube was placed in a magnetic separator till beads were separated from the liquid. Supernatant was removed. The beads were washed by adding 100µl of washing solution (85% ethanol) and incubated at room temperature for 1 minute. The washing step was repeated to ensure all contaminants had been removed. 40µl of elution buffer was added to the tube to elute the purified product and again incubated at room temperature for 1 minute. Tube was placed in the magnetic separator till the beads were separated from the liquid. Supernatant was shifted in another tube which has the purified PCR product. Sequencing procedure was then applied on the elute.

3.8.4 Capillary Electrophoresis

40µl of purified product was poured in the 96-well plate and sealed with septa. Sequencing was carried out in 3500XL Genetic Analyzer. Resultant data was generated in the form of ABI format and exported for further analysis.

3.9 Data Analysis

3.9.1 Sequence Editing

Normally automated DNA sequencers generate low quality reads at sequencing primer sites and at the end of longer sequence run. These sequences can't be excluded and distort the sequence assembly and subsequently the sequence analysis. For that purpose, the raw data generated by 3500XL was trimmed in the Sequencher software to remove low quality or ambiguous data and make a final FASTA file.

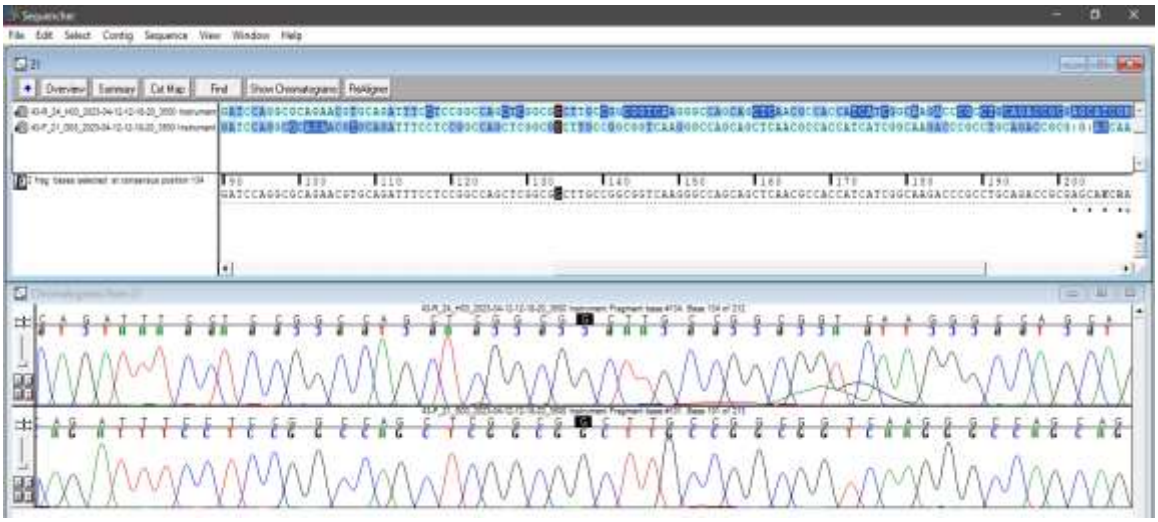

Figure 3.3. *Electropherogram of sequences for the editing of samples in Sequecher*

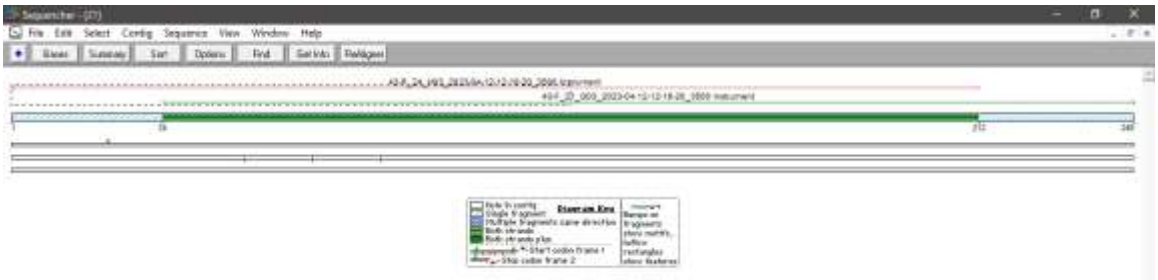

Figure 3.4 *Primers overlapped in Sequencher 4.9* software.

3.9.2 Alignment & Phylogenetic Analysis

To analyze the phylogenetic history of the sequenced isolates, first the sequences were BLASTed against the 16srRNA GenBank database. 18 sequences were selected for phylogenetic analysis. Reference type strains of the bacteria having greater query coverage and percentage identity in the BLAST results were downloaded from ATCC and BacDive in the FASTA format. Escherichia coli ATCC 11775 was used as an outgroup. For alignment, all the sequences along with the type sequences were imported into Mega 11 and aligned using Muscle. The gaps and extra sequences were trimmed from the aligned sequences. The resulting alignment can be seen in the figures.

Figure 3.5 *Alignments of query sequences and type strains in MEGA software.*

The resulting alignment was then exported in the Mega format for the construction of the phylogenetic tree. A neighborhood joining tree was constructed using Kimura 2 model. The analysis preference can be seen in figure.

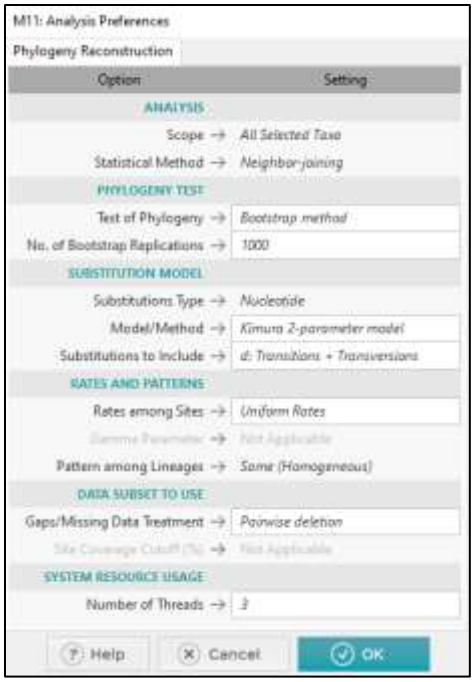

Figure 3.6 *Analysis Preferences of the Phylogenetic Tree Construction*

RESULTS

4.1 Demographic Distribution of Patients

A total of 28 sample of patients who were suffering from Functional Dyspepsia were taken between the period of December 2022 to March 2023 from Holy Family Hospital Rawalpindi. Out of these 28 patients, 13 (46%) were female and 15 (54%) were male. The patients lay between the age of 20 and 60. None of the patients was on any course of antibiotics or proton pump inhibitors when collecting the samples.

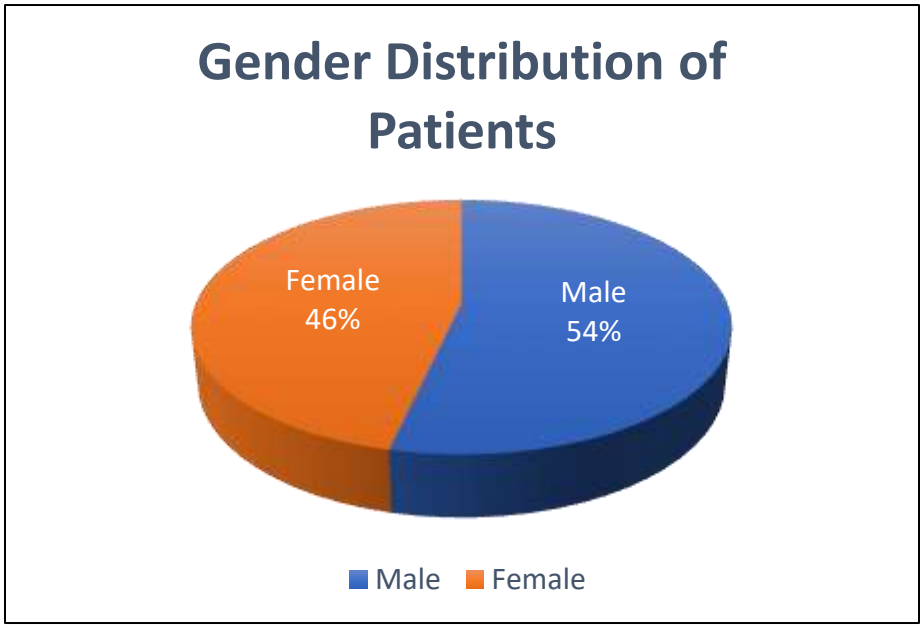

Figure 4. 1 Gender Distribution of Patients. *Out of 28 patients, 13 (46%) were female and 15 (54%) were male.*

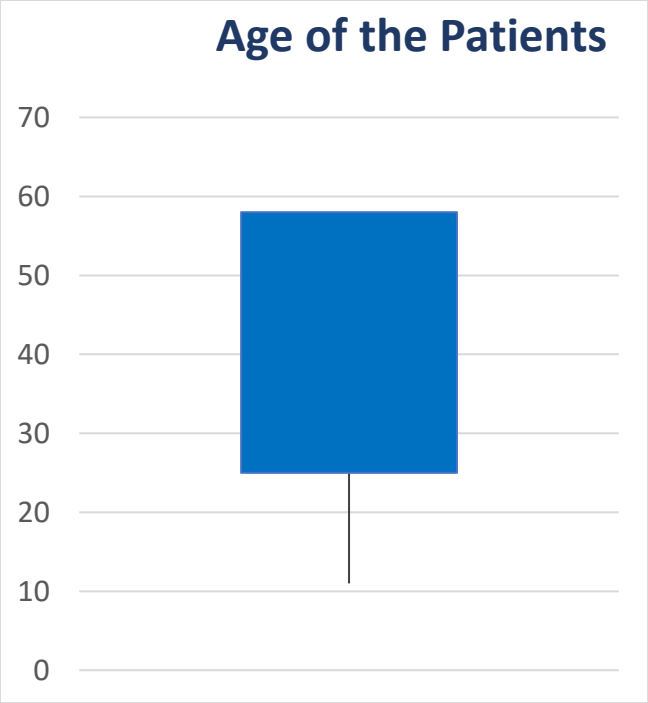

Figure 4. 2 *Age distribution of patients. The age of the patients lies between 20 and 60.*

4.2 Morphological Characterization of Bacteria

Firstly, bacterial colonies were identified on the basis of their morphological characteristics. A total of 60 colonies were obtained from the culturing of 28 biopsy samples which belong to 25 isolates upon the initial identification tests. The isolated microorganisms were differentiated based on their morphology (shape, colony formation, size and color).

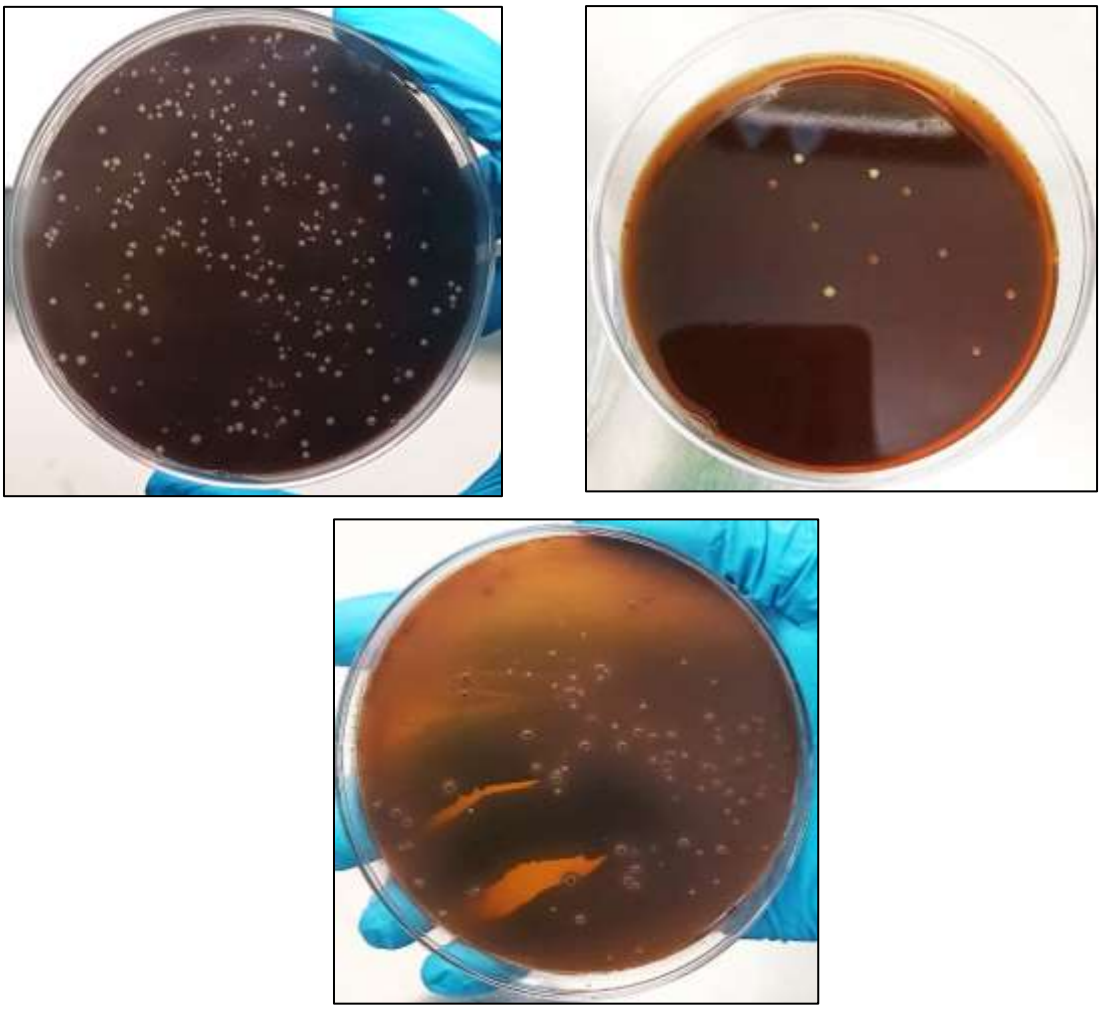

Figure 4. 3 *Morphological Characterization of bacterial isolates: This figure depicts the growth of different bacterial colonies on Blood Agar Plates Inoculated with biopsy samples.*

4.3 Bacterial Isolation

The 25 isolates were then streaked on blood agar repeatedly to obtain pure colony culture for further analysis of the samples.

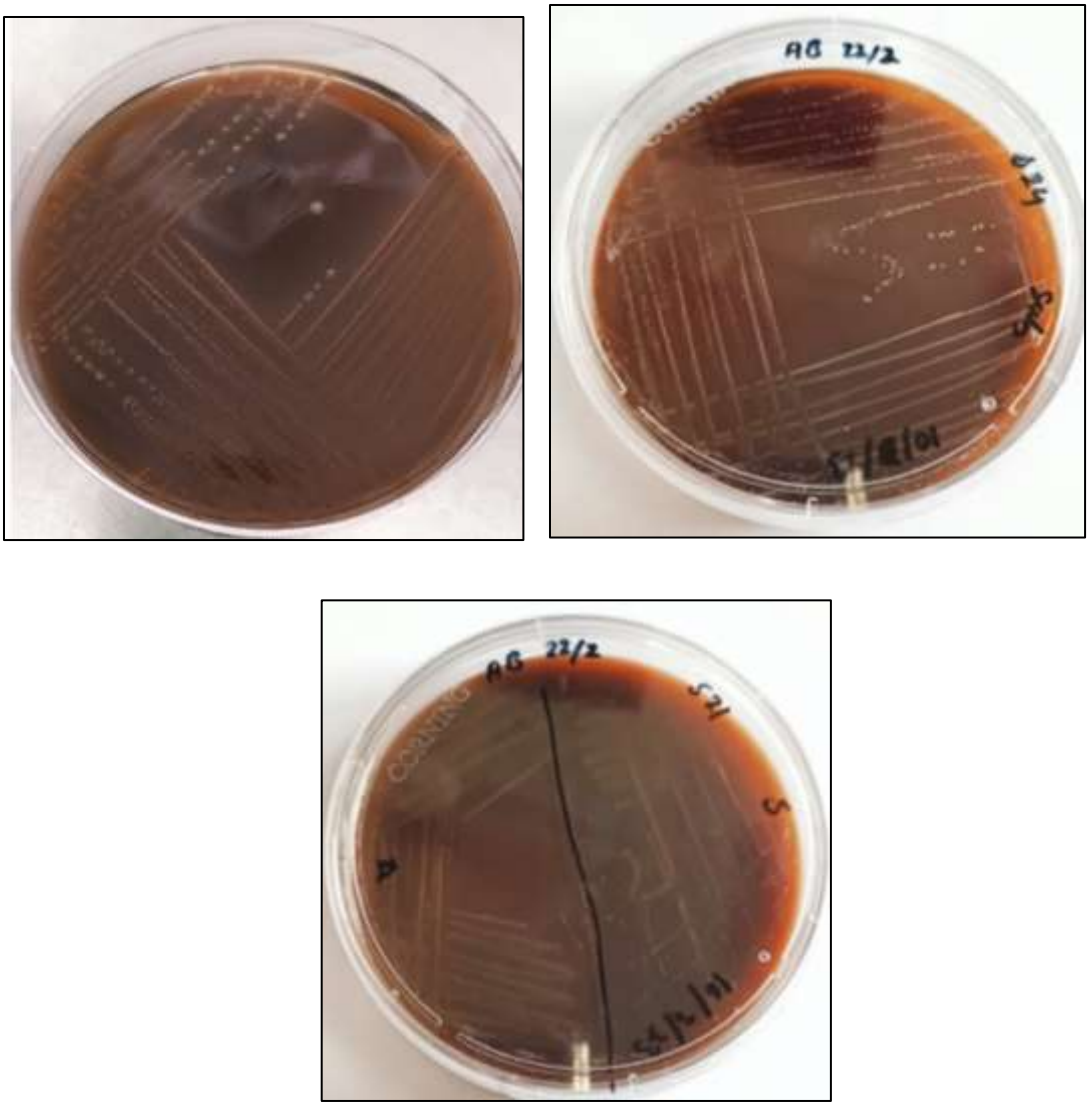

Figure 4. 4 *Pure Culture of Isolated Colonies picked from reference plates.*

4.4 Analyzing Colony PCR by Gel Electrophoresis

Colony PCR was done by lysing the bacterial colony by dissolving it in dH_2O and heating it at high temperature (95ºC). After PCR amplification, the DNA template was run on 2% agarose gel for quality assessment of DNA. Primers of the template DNA were annealed

at 58ºC and appeared as bright bands of approximately 1500bp which covers the whole 16srRNA gene.

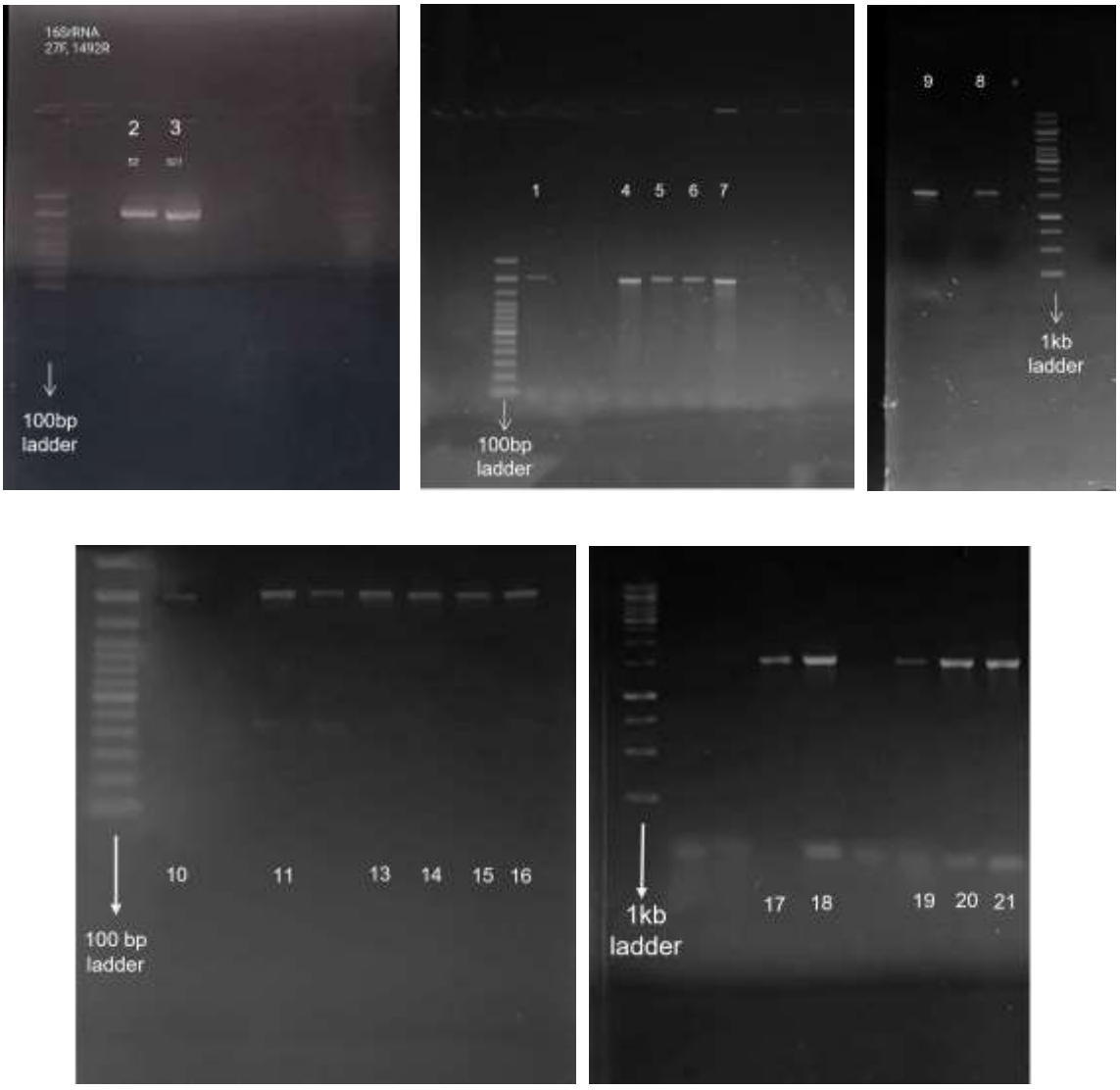

Figure 4. 5 *Agarose Gel Electrophoresis Representation of 16srRNA gene amplification of bacterial colony isolates.~1500bp long amplicon on 1X TAE 2% agarose gel*

4.4 Phylogenetic Analysis

The phylogenetic tree was constructed using the following reference type strains from ATCC and BacDive against query sequences:

Lacticaseibacillus casei	***ATCC 393***
Lacticaseibacillus paracasei subsp. paracasei	***ATCC 25303***
Lentilactobacillus buchneri	***ATCC 4005***
Lactobacillus buchneri	***BacDive***
Lactobacillus rhamnosus	***LC145553.1***
Lactobacillus casei	***LC064894***
Lactobacillus buchneri	***LC064887***
Lactobacillus paracasei subsp. paracasei	***LC096209***
Microbacterium sp.	***ATCC TSD-335***
Microbacterium testaceum	***ATCC 15829***
Pseudoarthrobacter chlorophenolicus	***ATCC 700700***
Arthrobacter scleromae	***BacDive***
Arthrobacter phenanthrenivorans	***BacDive***
Arthrobacter chlorophenolicus	***BacDive***
Arthrobacter phenanthrenivorans	***AM176541***

Table 4. 1 *Type strains taken from databases (GenBank, ATCC and BacDive) for phylogenetic analysis.*

For the phylogenegtic analysis of the 16srDNA bacterial isolates, neighborhood-joining tree was made using the Kimura-2 model in MEGA 11. 7 (BH13, BH26, BH1, BH11, BH9, BH21 & BH12) of the query sequences clustered around *Lactobacillus paracasei*, the 2nd cluster of 4 query sequences (BH27, BH4, BH20. BH5) was made around *Arthrobacter phenanthrevorans*. BH24 was seen to be clustered around *Microbacterium*. While 5 of the query sequences (BH18, BH8, BH25, BH15, BH16) were clustered around *Arthrobacter*.

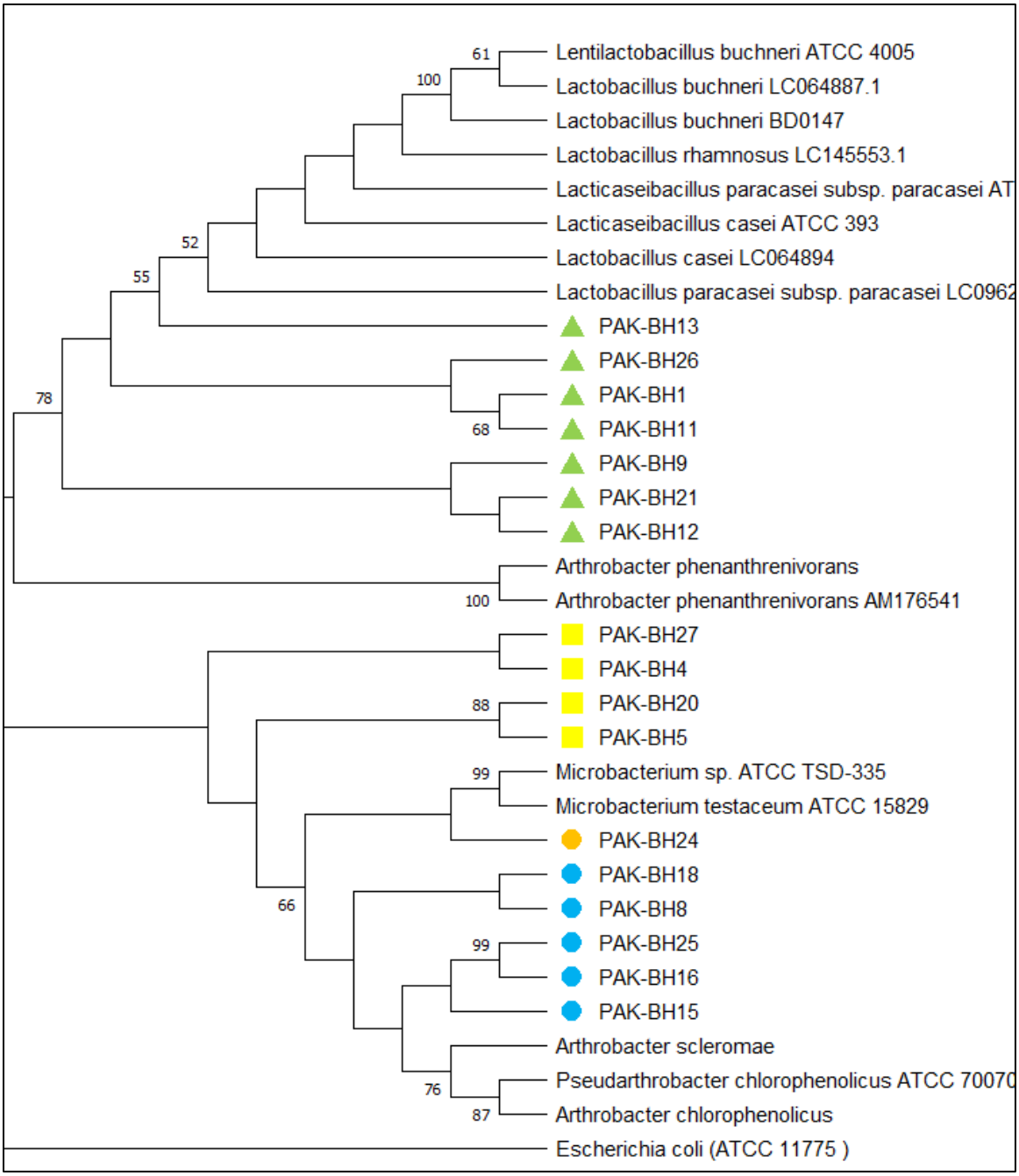

Figure 4. 6 A phylogenetic tree representative of all the 16srDNA sequences of bacterial isolates. *Evolutionary history was inferred using Kimura 2 model in Neighborhood Joining Tree. The frequency of the in which relevant taxa clustered together in the bootstrap value of 1000 is given on the side.*

On phylum level, the identification of the bacterial isolates falls into two categories: Firmicutes and Actinobacteria. 41% of the total samples fall under Firmicutes phylum and the remaining 59% in Actinobacteria.

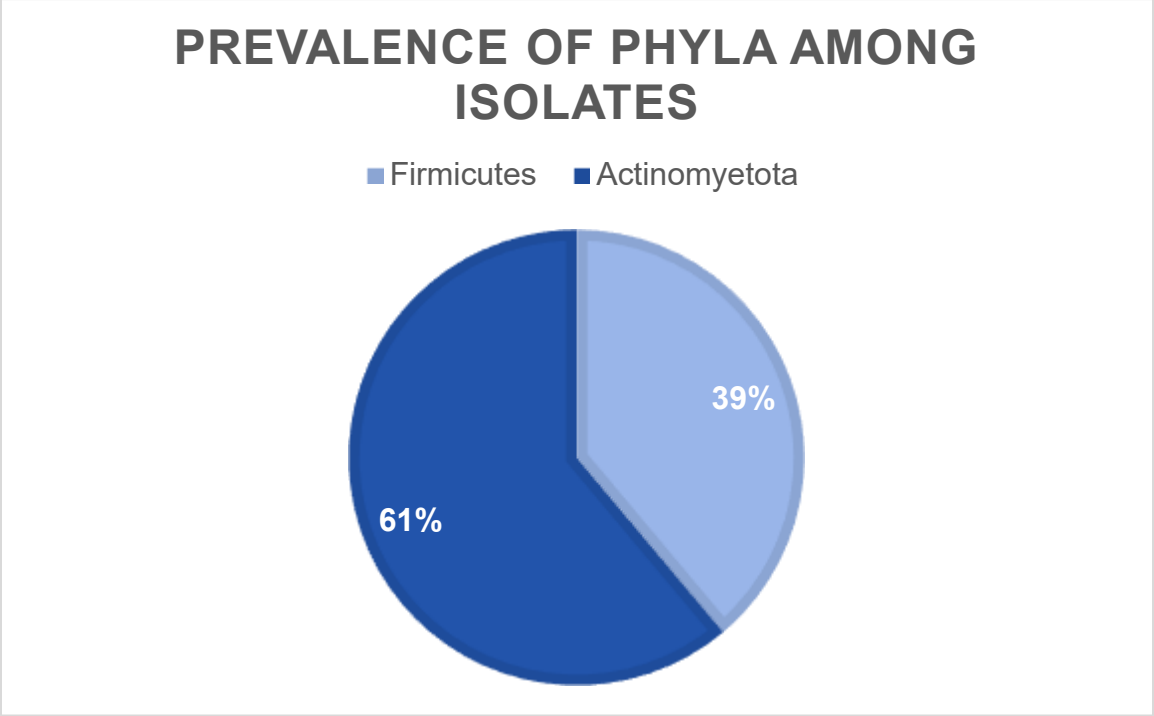

Figure 4. 7 Distribution of bacterial isolates in different phylum. *Out of 17 bacterial isolates 10 (58.8%) belong to the phylum Actinobacteria and 7 (41.2%) belong to Firmicutes.*

As 16srRNA could only give clear information regarding genus identification of bacteria. On the genus level, *Lactobacillus*, *Arthrobacter* and *Microbacterium* were found to be prevalent among functional dyspepsia patients.

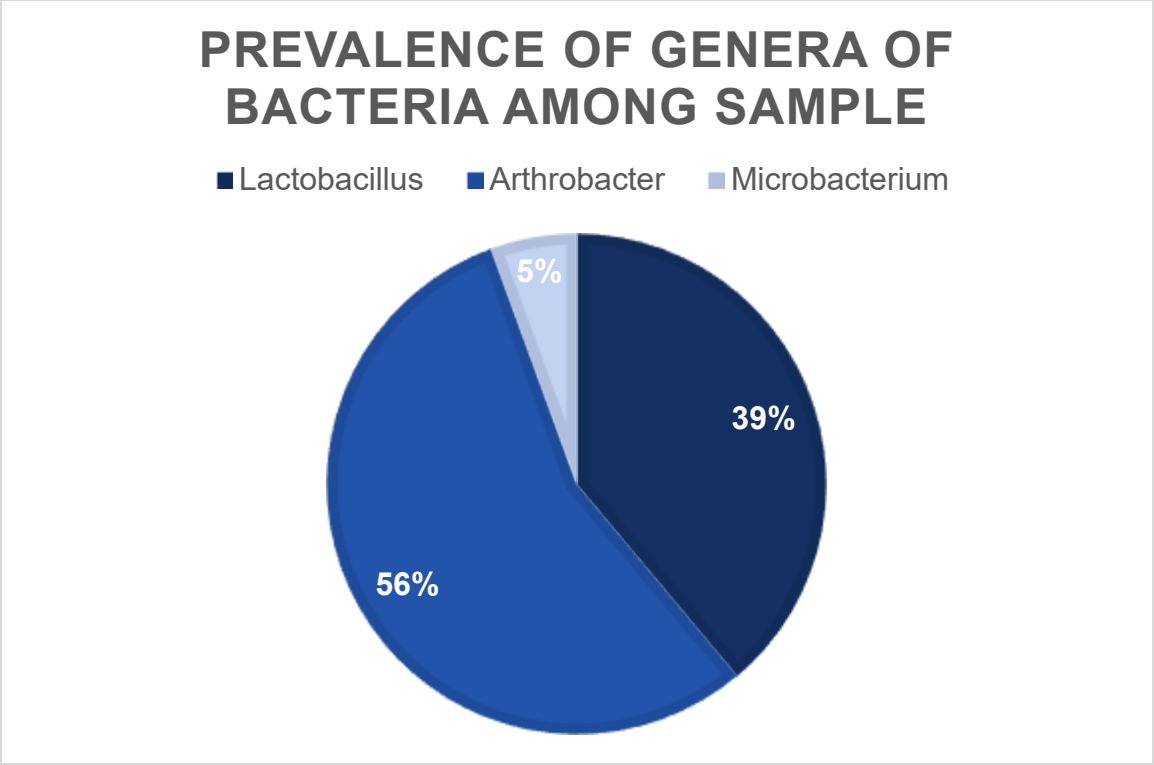

Figure 4.8 Distribution of bacterial isolates of bacteria on genus level. *Arthrobacter was in high prevalence constituting 56% of the total samples, along with Lactobacillus (39%) and Microbacterium (5%).*

DISCUSSION

The gut microbiota constitutes a diverse collection of bacteria that reside across the entirety of the mammalian gastrointestinal tract. Emerging research indicates that the gut mucosa-associated microbiota (MAM) is a leading candidate in the pathogenesis process of functional dyspepsia (FD) (Sterbini et al., 2016). Current study examines the correlation between gastrointestinal symptoms in functional dyspepsia (FD) and the attributes of the gastrointestinal mucosal-associated microbiota (MAM) of the stomach among Pakistani population. The primary objective of our study was to conduct a comprehensive analysis of the microbial population residing in stomach of individuals diagnosed with functional dyspepsia. First the samples were isolated with culturing techniques and identified with the help of V4 16srRNA gene sequencing and phylogenetic analysis. The investigation of the mucosal microbiota of stomach using 16srRNA sequencing highlights the notable prevalence of phylum *Actinobacteria* and Firmicutes. Phylogenetic analysis revealed the prominence of genera such as Arthrobacter, *Lactobacillus* and *Microbacterium* in the gastric mucosa community.

Firmicute phylum plays an important role in maintaining the gastrointestinal barrier which is involved in various pathophysiological processes. The genus Lactobacillus, which has been extensively studied as a probiotic, has been identified as an important aspect of the mucosal-associated microbiota in patients diagnosed with functional dyspepsia (FD). Findings of this study are consistent with previous research indicating the potential advantages of *Lactobacillus* genus in maintaining gastrointestinal homeostasis and modulating immune response. The prevalence of Lactobacillus in the mucosal environment of patients with FD suggests a potential role in attenuating inflammation and enhancing

the integrity of gastrointestinal barrier (Qin et al., 2022). Previous studies reported prevalence of *Firmicutes* in the gastrointestinal microbiota including *Lactobacillus.* Qiu et al.reported an increased prevalence of *Firmicute* phylum by sequencing samples from feces of rats' model of functional dyspepsia (FD) through high throughput 16srDNA technology. Another mucosa associated study of microbiota of the upper gastrointestinal tract, the individual diagnosed exhibited a notable elevation in the phylum *Firmicutes* in the GI tract in comparison to healthy control groups. While there was no significant difference observed in the MAM α-diversity between the two groups, there was a notable difference in β-diversity. Specifically, the phylum Firmicutes exhibited an increase in FD patients throughout all extracted biopsy samples (Fukui et al., 2020). Subsequent inquiries may focus on the distinct Lactobacillus species present and their prospective therapeutic implications in the management of symptoms associated with functional dyspepsia.

Although there is a majority of reports on the presence of *Actinobacteria* phylum, however, the presence of *Arthrobacter* and *Microbacterium*, which are often not usually found in the gastrointestinal system, was unexpectedly observed in the mucosal samples of individuals diagnosed with functional dyspepsia. The aforementioned genera have been recognized for their origins in soil and the environment, prompting questions regarding their presence in the gastric mucosa. Although the precise mechanisms driving their colonization within are not fully understood, it is plausible to consider that these bacteria may be delivered by food or water reservoirs. Furthermore, their abundance might be associated with altered stomach microenvironment in functional dyspepsia patients, providing them with an ecological niche to thrive. Investigating the potential interactions between Arthrobacter, *Microbacterium*, and resident gastric microbes could offer insights into their role in

dyspeptic conditions. The occurrence of these genera gives rise to thought-provoking inquiries on their influence on the pathophysiology of functional dyspepsia. The findings of this study have important useful implications, which necessitate further investigation through mechanistic research. These studies aim to explore potential connections between these genera and the gastrointestinal symptoms that are commonly observed in individuals with functional dyspepsia.

Although this study provides valuable insights, it is important to recognize certain limitations. The cross-sectional design of our research precludes the establishment of causality between microbial composition and functional dyspepsia. Longitudinal studies are essential for gaining a more comprehensive understanding of the dynamic fluctuations in mucosal-associated microbiota over a period of time, as well as their potential relationship with the severity of symptoms and the effectiveness of treatment interventions. Moreover, the exploration of other unknown taxa and species in relation to functional dyspepsia presents a promising option for future research. The culmination of our findings suggests a multifaceted microbial landscape in the gastric mucosa of functional dyspepsia patients, wherein *Lactobacillus*, *Arthrobacter*, and *Microbacterium*, exerts influence which should be further investigated in a comprehensive manner.

CONCLUSION

To summarize, the current study implemented 16srRNA sequencing to investigate the composition and potential significance of mucosal associated microbiota of stomach in individuals suffering from functional dyspepsia. The investigation yielded compelling results, particularly highlighting the abundance of *Arthrobacter* and *Lactobacillus* at the taxonomic ranking of genus, alongside Firmicutes and *Actinobacteria* at phylum level.

The presence of *Lactobacillus* in the mucosal microbiota may suggest the potential involvement of beneficial bacteria in mitigating dyspeptic symptoms. Probiotic properties of *Lactobacillus* highlight the importance of conducting more research to elucidate its specific mechanisms of interaction with the gastric mucosa. Moreover, the identification of *Arthrobacter* and *Microbacterium*, which are considered as ambient bacteria, may prove that alteration of microbiota of stomach due to functional dyspepsia provides a favorable environment for these bacteria which can be which can be transmitted through contaminated food or water. Exploring their metabolic and functional traits could uncover unforeseen roles in functional dyspepsia pathogenesis.

The phylum level identification of *Firmicutes* and *Actinobacteria* offers valuable insights into broader shifts within the microbial communities of functional dyspepsia patients. The examination of the potential consequences of these variations in phyla on gut-brain connections, inflammation, and digestion presents promising avenues for further investigation. As our comprehension of the role of the gut microbiota in maintaining health and contributing to disease progresses, the importance of these results at the phylum level in relation to functional dyspepsia becomes more pertinent.

Dysbiosis has been suggested to have a potential association with the production or worsening of symptoms in a specific group of patients with functional dyspepsia, through various putative pathways. Recognizing the limitations of existing data in establishing a definitive causal relationship between the development of functional dyspepsia (FD) and specific factors, it is evident that these findings serve as a valuable foundation for future investigations aimed at enhancing our understanding of the role of the microbiota in FD.

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Isolation and Characterization of Mucosa Associated Microbiota (MAM) of Stomach in Functional Dyspepsia Patients Among Pakistani Population

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