CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Implications of Gut Microbial Variations in Autism Spectrum Disorder

by

Sobia Khurshid

A dissertation submitted in partial fulfillment for the degree of Doctor of Philosophy

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2025

Implications of Gut Microbial Variations in Autism Spectrum Disorder

By

Sobia Khurshid

(DBS181001)

Dr. Muhammad Faheem, Senior Researcher

Mayo Clinic, Florida, USA

(Foreign Evaluator 1)

Dr. Sajid Hussain, Professor

University of Cologne, Germany

(Foreign Evaluator 2)

Dr. Syeda Marriam Bakhtiar

(Research Supervisor)

Dr. Syeda Marriam Bakhtiar

(Head, Department of Bioinformatics and Biosciences)

Dr. Sahar Fazal

(Dean, Faculty of Health and Life Sciences)

DEPARTMENT OF BIOINFORMATICS AND BIOSCIENCES

CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY

ISLAMABAD

2025

I dedicate this dissertation to my family, Specially to my father and mother whose unconditional love, unwavering support, and endless encouragement have been my pillars of strength throughout this challenging journey. Their belief in me and sacrifices made have been the driving force behind my success. I am grateful for their patience, understanding, and constant motivation. This achievement is a testament to their unwavering faith in my abilities. I dedicate this work to them as a token of my deepest appreciation and love.

CAPITAL UNIVERSITY OF SCIENCE & TECHNOLOGY
ISLAMABAD
Expressway, Kahuta Road, Zone-V, Islamabad
Phone:+92-51-111-555-666 Fax: +92-51-4486705
Email: info@cust.edu.pk Website: https://www.cust.edu.pk

CERTIFICATE OF APPROVAL

This is to certify that the research work presented in the dissertation, entitled "**Implications of Gut Microbial Variations in Autism Spectrum Disorder**" was conducted under the supervision of **Dr. Syeda Marriam Bakhtiar**. No part of this dissertation has been submitted anywhere else for any other degree. This dissertation is submitted to the **Department of Bioinformatics & Biosciences, Capital University of Science and Technology** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of **Biosciences**. The open defence of the dissertation was conducted on **February 03, 2025.**

Student Name : Sobia Khurshid (DBS181001)

The Examination Committee unanimously agrees to award PhD degree in the mentioned field.

Examination Committee :

(a) External Examiner 1: Dr. Sobia Tabassum
Professor
IIU, Islamabad

(b) External Examiner 2: Dr. Bashir Ahmad
Associate Professor
IIU, Islamabad

(c) Internal Examiner : Dr. Arshia Amin Butt
Associate Professor
CUST, Islamabad

Supervisor Name : Dr. Syeda Marriam Bakhtiar
Associate Professor
CUST, Islamabad

Name of HoD : Syeda Marriam Bakhtiar
Associate Professor
CUST, Islamabad

Name of Dean : Dr. Sahar Fazal
Professor
CUST, Islamabad

AUTHOR'S DECLARATION

I, **Sobia Khurshid** (Registration No. **DBS181001)**, hereby state that my dissertation titled, '**Implications of Gut Microbial Variations in Autism Spectrum Disorder**' is my own work and has not been submitted previously by me for taking any degree from Capital University of Science and Technology, Islamabad or anywhere else in the country/ world.

At any time, if my statement is found to be incorrect even after my graduation, the University has the right to withdraw my PhD Degree.

(**Sobia Khurshid**)

Dated: 03 February, 2025

Registration No : DBS181001

PLAGIARISM UNDERTAKING

I solemnly declare that research work presented in the dissertation titled "**Implications of Gut Microbial Variations in Autism Spectrum Disorder**" 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 dissertation has been written by me.

I understand the zero-tolerance policy of the HEC and Capital University of Science and Technology towards plagiarism. Therefore, I as an author of the above titled dissertation declare that no portion of my dissertation 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 dissertation even after award of PhD Degree, the University reserves the right to withdraw/ revoke my PhD degree and that HEC and the University have the right to publish my name on the HEC/ University Website on which names of students are placed who submitted plagiarized dissertation.

(**Sobia Khurshid**)

Dated: 03 February, 2025

Registration No : DBS181001

List of Publications

It is certified that following publication(s) have been made out of the research work that has been carried out for this dissertation:-

1. **Sobia Khurshid** and **Syeda Marriam Bakhtiar**, "Analysis of Alterations in Fecal Metagenomic Profile in Autism Spectrum Disorder", *J Popl Ther Clin Pharmacol*, vol. 31, no. 6, pp. 987–998, Jun. 2024.

(Sobia Khurshid)
Registration No: DBS181001

Acknowledgement

First and foremost, all attributes to Almighty ALLAH, The Gracious and The Merciful, who blessed me with the perfect circumstances throughout my life, and bestowed me with the potential, ability and an optimal environment to accomplish my PhD project. I owe my deep gratitude to the teachings of Holy Prophet Hazrat Muhammad (SAW), which are a source of inspiration for every aspect of life, for the entire creation. His teachings focusing on the seeking of knowledge inspired me and gave me strength to undertake this research work. I present my heartfelt thanks to the Dean Dr. Sahar Fazal, Faculty of Health and Life Sciences, Capital University of Science and Technology, Islamabad, who is a very kind and dedicated teacher. She is maxim for all students.

I find it a perfect opportunity to express my feelings of deep thanks and admirations to a person of extreme dedication in the field of Biosciences, my supervisor, Associate Professor Dr. Syeda Marriam Bakhtiar, who provided me with the opportunity to work in her excelled and utmost supervision. Her patience, valuable suggestions and kind interest enabled me to complete my research work. She is an inspiration for me and I am honored to be in her supervision. May Almighty ALLAH bless her with perfect health and potential to carry on her valuable work, Ameen.

Completion of research work necessitates peaceful working environment, instruments' management and pleasant company. I wish to express my heartiest thanks to my lab fellows:Naveed Iqbals Soomro and Muhammad Qasim for their perfect company and cooperation. I would like to express my sincere thanks to my seniors and juniors for their support and company.

I wish to express feelings of love and thanks to friends for their love, support, valuable company, and unlimited moral support during my research work and write up. No words in any dictionary can acknowledge the sacrifices, love and moral support given to me by my parents. My studies and success is wholly and solely attributed to my parents. Their desires inspire me to excel further in my studies. Special acknowledgments to my husband who has always been there for me during all ups and downs,

and without him, I could never accomplish my PhD degree. I owe my deep admiration to my brothers and sisters who supported me not only during my research work, but throughout my life.

In the end I want to present my indebtedness to all those hands that prayed for my betterment and serenity.

(Sobia Khurshid)

Abstract

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition marked by significant impairments in social interaction, communication deficits, and the presence of restricted, repetitive patterns of behavior or activities. In recent years, the prevalence of ASD has risen, making it a major public health concern. While the etiology of ASD remains incompletely understood, it is believed to result from a combination of genetic, epigenetic, and environmental factors. An emerging body of research suggests that alterations in gut microbiota may also play a critical role in the pathophysiology of ASD. This association is further supported by the frequent occurrence of gastrointestinal disturbances in individuals with ASD and growing evidence of the gut-brain axis—a bi-directional communication pathway between the central nervous system and the gastrointestinal tract.

The primary objective of this study is to investigate the gut microbial composition in children with ASD compared to neurotypically developing (control) children. The study seeks to elucidate the specific microbial differences and their potential contributions to the pathophysiology of ASD, focusing on the role of microbial dysbiosis in these children. Given the inconsistent findings in previous studies and the lack of research on ASD and gut microbiota in the Pakistani population, this study also aims to address this gap by examining the gut microbial profiles in Pakistani children with ASD.

The study was conducted in three distinct phases. In the first phase, a comprehensive meta-analysis was conducted to assess the relative abundance of specific gut microbial phyla and genera in children with ASD compared to healthy controls. A systematic search of electronic databases was carried out to identify relevant studies published up to July 2023. A total of 21 studies were included in the meta-analysis, encompassing data from 773 children with ASD and 629 neurotypically developing controls. The meta-analysis was performed using RevMan 5.3 software. The results revealed significant differences in the gut microbial composition between ASD and control groups. Children with ASD exhibited a higher relative abundance of *Clostridium* and *Faeca-*

libacterium compared to healthy controls, while *Bifidobacterium* and *Coprococcus* were found in lower relative abundance in the ASD group. Despite these findings, the literature demonstrates a lack of consensus on which specific bacterial genera are consistently altered in ASD versus control groups. This inconsistency highlights the need for further research to clarify these microbial differences, particularly in understudied populations such as children in Pakistan.

The second phase of the study involved the direct assessment of gut microbial composition in a small cohort of children from Pakistan. Two children with ASD and two neurotypically developing (control) children were enrolled in the study. Information on social, demographic, and dietary factors was collected using detailed questionnaires, ensuring that these variables were controlled for during the analysis.

Fecal samples were collected from each child and subjected to 16S rRNA sequencing to determine the microbial community composition. The results revealed notable differences in gut microbial diversity between ASD and control children. Alpha diversity, which measures the richness and evenness of microbial species within a sample, was significantly lower in children with ASD compared to controls, indicating reduced microbial diversity. Beta diversity, which reflects differences in microbial community structure between groups, showed distinct clustering patterns for ASD and control children, suggesting that the overall microbial composition differed between the two groups.

At the phylum level, the relative abundance of *Proteobacteria* was significantly reduced in ASD children compared to controls. At the genus level, *Lachnospiraceae UCG-004* was found to be less abundant in ASD children. These findings provide evidence of gut microbial dysbiosis in Pakistani children with ASD, consistent with observations in other populations, but also reveal unique microbial signatures specific to this cohort.

The third phase of the study focused on identifying metabolites produced by the gut microbial genera that were prioritized based on the findings from both the meta-analysis and the 16S rRNA sequencing data. Electronic databases were searched to identify metabolites produced by the genera of interest, including *Clostridium, Faec-*

alibacterium, Bifidobacterium, *Coprococcus* and *Lachnospraceae UCG004*. The identified metabolites included L-Acetone, D-lactic acid, Vinylacetyl CoA, and 10- formyltetrahydrofolate. These metabolites were then mapped to their respective metabolic pathways using Metaboanalyst. The analysis revealed key metabolic pathways that are potentially influenced by the altered gut microbial composition in ASD children, further implicating microbial dysbiosis in the metabolic disturbances observed in ASD.

This study provides comprehensive evidence of gut microbial dysbiosis in children with Autism Spectrum Disorder. The results from the meta-analysis, 16S rRNA sequencing, and metabolite analysis indicate significant differences in the gut microbiota and their metabolic output between ASD and neurotypically developing children. Specifically, ASD children were found to have lower microbial diversity and distinct microbial signatures, with higher relative abundances of *Clostridium* and *Faecalibacterium* and lower abundances of *Bifidobacterium* and *Coprococcus*. The presence of reduced alpha diversity and altered beta diversity in ASD children underscores the potential role of gut microbial imbalances in the disorder.

The identification of key microbial metabolites and their associated metabolic pathways further supports the hypothesis that gut microbiota may influence the neurodevelopmental and metabolic features of ASD. These findings suggest that therapeutic interventions targeting the gut microbiota, such as the administration of prebiotics and probiotics, may hold promise for alleviating ASD symptoms by restoring microbial balance and improving gut-brain communication.

This research represents an important step toward understanding the role of the gut microbiome in Autism Spectrum Disorder, with implications for future studies and potential treatments aimed at modulating gut microbial composition as a therapeutic strategy for ASD.

Contents

List of Figures

List of Tables

Abbreviations

ABA	Applied Behavior Analysis
ACTH	Adrenocorticotropic Hormone
ADHDA	Attention Deficit Hyperactivity Disorder
ADNP	Activity-dependent neuroprotector homeobox
AMP	Adenosine Monophosphate-activated
ANKRD 11	Ankyrin repeat domain 11
ARID1B	AT-rich interaction domain 1B
ASD	Autism Spectrum Disorder
ASXL3	ASXL Transcriptional Regulator 3
ATRX	ATRX Chromatin Remodeler
AUTS2	Autism susceptibility candidate 2
BBB	Blood Brain Barrie
BP	Base Pair
CDC	Centre for Disease Control
CDKL5	Cyclin-dependent kinase-like 5
CHD2	Chromodomain helicase DNA binding protein 2
CHD7	Chromodomain helicase DNA binding protein 7
CHD8	Chromodomain helicase DNA binding protein 8
CI	Confidence Interval
CNS	Central Nervous System
CNS	Central Nervous System
CREBBP	CREB-binding protein

CRH	Corticotropin releasing Hormone
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
DYRK1A	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
EHMT1	Euchromatic histone-lysine N-methyltransferase 1
FAAs	Free Amino Acids
FMR1	Fragile X mental retardation 1
FOXG1	Forkhead box G1
FOXP1	Forkhead box P1
FOXP2	Forkhead box P2
GABA	Gamma Amino Butyric Acid
GABA	Gamma-Amino butyric acid
GI	GastroIntestinal
GIS	Gastro Intestinal Symptoms
GLP	Glucagon-Like Peptides
GPR	G protein-coupled receptor
HPA	Hypothalamic-pituitary-adrenal
KEGG	Kyoto Encyclopedia of Genes and Genomes
MBD5	Methyl-CpG binding domain protein 5
MECP2	Methyl CpG binding protein 2
MED13L	Mediator complex subunit 13-like
MIA	Maternal Immune Activation
MIA	Mental Immune Activation
NADH	Nicotinamide adenine dinucleotide
NF1	Neurofibromin 1
NFKB	Nuclear Factor Kappa B
NT	NeuroTypically
OTUs	Operational Taxonomic Units
PBS	Phosphate-Buffered Saline

PCA	Principal Coordinate Analysis
POGZ	Pogo transposable element with ZNF domain
PRISMA	Prefered Reporting Items for Systemetaic Reviews and Meta-analysis
PTEN	Phosphatase and tensin homolog
QIME	Quantitative Insights into Microbial Ecology
RA	Relative abundance
RAI1	Retinoic Acid Induced 1
RDP	Ribosomal Database Project
rRNA	Ribosomal Ribose Nucleic Acid
SCFAs	Short Chain Fatty Acids
SCFAs	Short Chain Fatty Acids
SE	Standard Erro
SETD5	SET domain containing 5
SMD	Standardized mean difference
SYNGAP1	Synaptic Ras GTPase activating protein 1
TBR1	T-box, brain 1
TCF4	Transcription factor 4
TLR4	Toll Like Receptors 4
TSC1/TSC2	Tuberous sclerosis 1/2
UBE3A	Ubiquitin protein ligase E3A
ZBTB20	Zinc finger and BTB domain containing 20

Symbols

%	Percentage
$	Dollar
£	Pound
$\geq$	Greater than or equal to
$<$	Less than
$>$	Greater than
$\leq$	Less than or equal to
$mmol/L$	Millimoles per Liter
mg/dL	Milligrams per Deciliter
g	Gram
α	Alpha
β	Beta
γ	Gamma
θ	Theta
λ	Lambda

Chapter 1

Introduction

1.1 Background of the Study

The prevalence of ASD has escalated in the past decade and 1 out of 36 children are being identified with ASD [1, 2]. Recently, the Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V) has been used to diagnose this disorder. Ideally, ASD must be diagnosed early in childhood but the diversity in symptoms, inadequacy of biomarkers, and shortcomings in diagnostic methods hinder the early diagnosis [3]. Some common signs and symptoms of ASD subjects are shown in the figure 1.1.

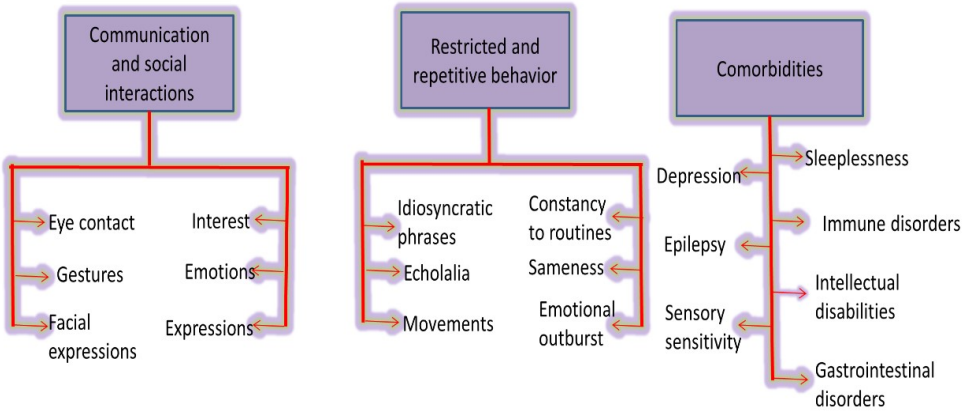

FIGURE 1.1: A diverse range of symptoms and phenotypes along with various comorbid health conditions depicted by Individuals with ASD.

Currently, no proper treatment is available for ASD populations except behavioral management therapies, educational interventions, and some drugs to alleviate the spectrum of symptoms linked with ASD. This only ensures the improvement in the quality of life of autistic individuals by relieving the symptoms associated with the disorder. However it imparts an economic burden on society, and the lack of proper treatment complicates the life of autistic populations [4].

The unavailability of proper drug protocol and the social, economic, and emotional concerns intrigue the researchers for an in-depth search to unravel the pathophysiology, diagnosis, and treatment of ASD.

The etiology of ASD remains obscure, with the involvement of genetic, epigenetic, and environmental factors, labeling it as a multifactorial disorder.

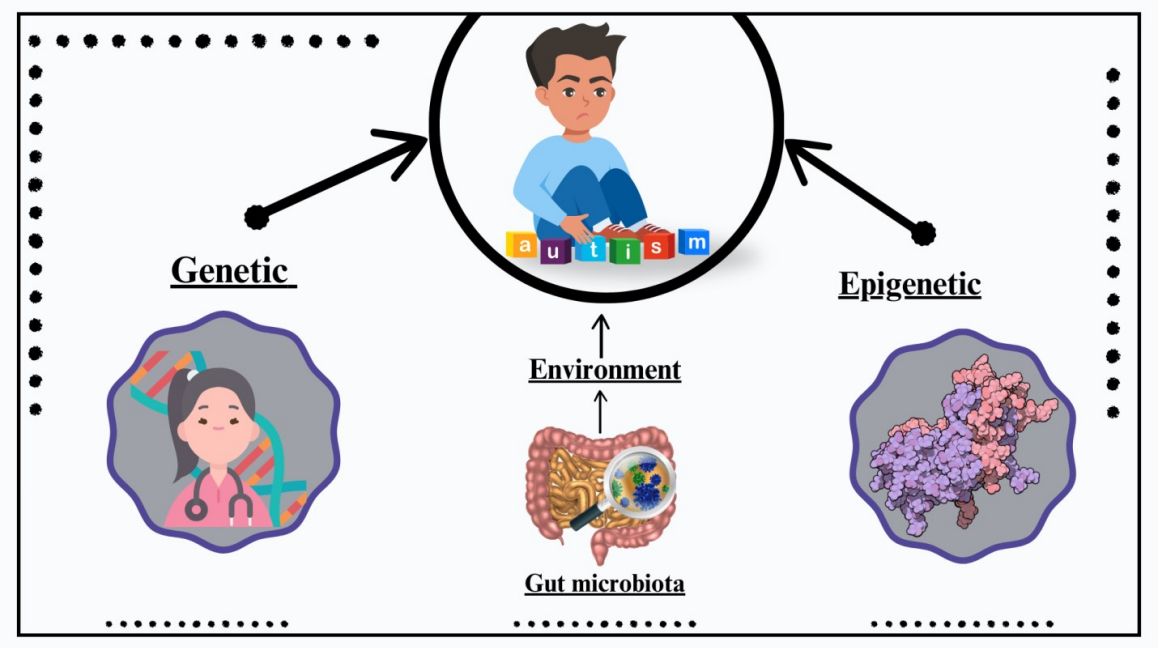

FIGURE 1.2: Risk factors in the etiology of autism spectrum disorder.

Various genetic components, de novo mutations, and copy number variations are associated with ASD, but the search for a main hub gene is still on the way. Moreover, the associated genetic changes cannot demonstrate the spectrum of phenotypes observed in ASD patients. Thus, the pathophysiology of ASD remains obscure, emphasizing the role of various genetic, epigenetic, and environmental factors, and quests for exploring the fine interactions between these various factors [5]. ASD individuals exhibit

a wide range of phenotypes of symptoms and various co-morbid health conditions are associated with them, the most common being gastrointestinal (GI) problems including vomiting, constipation, bloating, diarrhea, and abdominal pain. Studies report the direct connection between the severity of GI problems and ASD behaviors. This co-morbid medical condition emphasizes the possible role of microbes residing in the human gut [6].

Gut microbes have also been shown to affect the development of the brain through gut-brain interaction pathways. Gut-brain axis has also been linked to the biochemical and behavioral changes in the brain, primarily due to the varied composition of gut microbiota and its potentially harmful effects on the human brain. The varied composition of gut microbes causes gut dysbiosis which has shown a strong connection with neuropathological conditions [7].

Various symptoms associated with neurodevelopmental disorders have been linked to be caused by variations in the composition of gut microbiota. Moreover, gut microbes have been shown to possess therapeutic potentials for ASD [8] Various studies have shown the varied composition of gut microbiota in ASD when compared with healthy populations, and numerous species have been shown to cause microbial dysbiosis in ASD subjects. However, inconsistent results have been drawn by different studies regarding the gut microbial composition. Previous studies showed a lower percentage of *Bifidobacterium* and *Coprococcus*, and a higher relative abundance of the genera *Clostridium*, *Parabacteroides*, *Faecalibacterium*, Bacteroides, and *Phascolarctobacterium* in ASD affected children as compared to healthy controls [9].

Various proteins and metabolites are produced as secondary metabolites gut microbiota. These metabolites not only exert their effects in the local gut environment but also possess the ability to cross the Blood Brain Barrier (BBB) and enter into the circulation. Once in circulation, these metabolites possess the potential to reach the brain, where they not only interact with other proteins and alter metabolic pathways but also can effect gene expression. These gut metabolites also influence the metabolic pathways, altering their activities. The metabolites produced by the human gut microbiota are presumed to be one of the potent underlying mechanisms involved in the

pathophysiology of ASD [10]. Therefore studies focusing on gut microbial composition, and analysis of various metabolites produced by the gut microbes could help researchers to provide greater insight into the etiology of the multifactorial nature of ASD.

1.2 Gap Analysis

The pathophysiology of ASD is complex and obscure, involving multiple factors ranging from genetic, and epigenetic to environmental. One of the potent factors implicated in the etiology of ASD is the gut microbiota owing to the crucial functions performed by it, and manipulating the host gene expression and functions. Multiple studies have shown the variations in gut microbial composition in ASD individuals as compared to the neurotypically growing ones. Metabolites produced by gut microbiota affect the gut-brain physiology and are presumed to be involved in the underlying pathogenesis of ASD. However, the studies conducted to depict the variations in gut microbial composition in ASD individuals and controls show inconsistent results. Certain gut bacterial genera are reported to be highly abundant in ASD children than in controls but the same is reported as vice-versa in certain other studies. Moreover, the underlying caustive mechanism and pathways underpinning the involvement of gut microbiota in ASD are not fully comprehended. Thus, various lines of study show that gut microbiota is associated with ASD, but the exact mechanism behind the interaction between the two is unclear.

1.3 Problem Statement

This is an established fact that gut microbial variations exist in ASD children as compared to the controls, however, the results related to bacterial diversity are inconsistent therefore it is yet to be established which microbial species contribute to the onset of ASD or are associated with the severity of symptoms.

Thus, the underlying mechanisms governing the role of certain gut microbial species needs to be explored in order to delineate the pathophysiology of ASD.

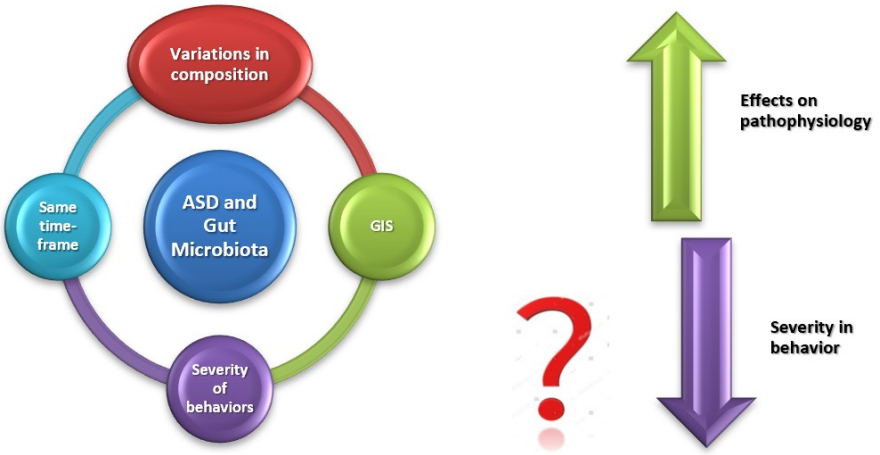

FIGURE 1.3: The schematic diagram to represent the problem statement of the study.

1.4 Proposed Solutions

The current study will is in effort to comprehend the variations of gut microbial composition in ASD individuals, In addition, statistically significant results will be generated to obtain consistent and reliable results regarding the alterations of gut microbial composition in ASD individuals as compared to the controls. Moreover, the mechanisms by which the microbial diversity contributes towards ASD is explored.

1.5 Research Questions

This research will answer the following questions:

Research Question 1

What is the level of gut microbial diversity in children with Autism Spectrum Disorder (ASD), and how does it differ from the diversity observed in neurotypical children?

This question aims to explore whether there are significant differences in the range and variety of microbial species present in the gut of ASD children compared to their neurotypical counterparts. It seeks to determine if gut microbial diversity is reduced, increased, or altered in specific ways in ASD populations.

Research Question 2

What are the key variations in the composition of gut microbiota in children with ASD compared to neurotypical Pakistani children, and how do these compositional changes relate to the clinical features of ASD?

This research question focuses on identifying specific gut microbial species or groups that differ in abundance between children with ASD and neurotypical children. It also seeks to investigate if these compositional changes are linked to the behavioral, cognitive, or gastrointestinal symptoms often observed in ASD, providing insights into the potential microbiome-related mechanisms of the disorder.

Research Question 3

How do metabolites produced by gut microbiota influence the microbiota gut-brain axis in children with ASD, and what is their role in the neurological and behavioral manifestations of the disorder?

This question addresses the functional aspect of the gut-brain connection by examining the role of microbial metabolites—chemicals produced by gut bacteria in modulating brain activity and behavior in ASD. The goal is to understand how these metabolites may contribute to the development or progression of ASD symptoms through interactions within the microbiota-gut-brain axis.

1.6 Aim of the Study

This study had been conducted with the aim to understand and elaborate the Microbiota Gut-Brain Axis alterations in ASD. The present research project quested for the

diversity of gut microbiota, the way microbial variations effects metabolic pathways and their connections to one another with respect to their implications in ASD.

1.7 Research Objectives for This Study

The main objectives of this study are discussed below:

1.7.1 Research Objective 1

To evaluate the diversity of gut microbiota in pediatric populations diagnosed with Autism Spectrum Disorder (ASD).

1.7.2 Research Objective 2

To characterize the compositional differences in gut microbiota among children with ASD.

1.7.3 Research Objective 3

To elucidate the functional dynamics of the microbiota-gut-brain axis in ASD by analyzing microbial-derived metabolites.

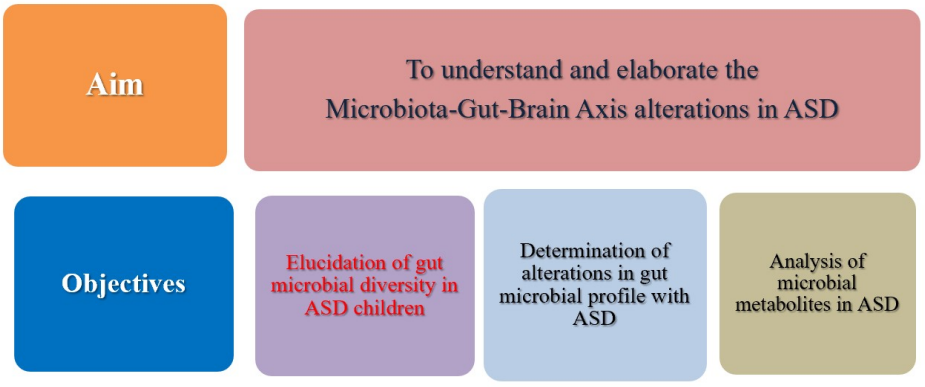

FIGURE 1.4: Pictorial representation of aims and objectives of current study.

1.8 Research Philosophy and Significance

The role of genes, epigenetics, and environmental factors in the etiology of ASD is well dicumented. Gut microbiota, however, has emerged as a potent risk factor among all due to the same time frame of development and maturation as the Central Nervous System (CNS), the evident variations in its composition, and its association with the severity of autistic symptoms, but how exactly it affects the pathophysiology is still unclear.

This study would explore the diversity of gut microbial profile in ASD children as well as analyze the interactions of gut microbiota in onset and severity of ASD. The study contributes towards the assessment of the gut microbial diversity in ASD children, exploration of gut microbial abundance in children with ASD, and prioritization of the metabolites produced by the gut microbes that could play some role in the onset or development of ASD. This study intrigues to explore the etiology of ASD, aiming to assist the researchers in figuring out the possibilities of treatment for this disorder. This work would not only elucidate the mechanism by which the gut microbiota is associated with the pathophysiology of ASD rather it will add more towards efficient diagnosis and treatment strategies. Moreover, the knowledge obtained by the current study can be employed by scientists to underpin the mechanisms regarding the probable roles played by gut microbes in other diseases.

1.9 Research Methodology

1.9.1 Exploration of Variations in Gut Microbial Composition

The variations in gut microbial composition in autistic children has been quested through meta-analysis. Meta-analysis refers to the process of systematically assessing the results of previous studies to derive statistically proven conclusions [11]. In

the current study, standard procedure of the meta-analysis has been adopted, forest plot of the included studies are constructed, and conclusive results are drawn regarding the variations in gut microbial composition in autistic children as compared to neurotypically developed individuals.

1.9.2 Analysis of Gut Microbial Diversity

To validate variations of gut microbes in autistic children of Pakistani origin, 16S rRNA gene-based metagenome analysis has been conducted. 16S rRNA gene has been extensively employed as a phylogenetic marker in metagenome analysis as it is present in all prokaryotes, its sequence changes slower over the time, possesses hypervariable regions namely V1-V9 with sufficient sequence diversity to classify the microbes, and the presence of conserved regions flanking the variable regions thus allow the designing of universal primers [11].

Steps including sample collection, DNA isolation, 16S rRNA sequencing, and sequence analysis using various computational tools have been proformed. After 16S rRNA gene sequencing, Operational Taxonomic Unit (OTU) based methods that resolve the sequencing errors by clustering the reads based on an already defined identity threshold into OTUs are used for profiling the microbial abundance in the sequenced data [12]. Detailed analysis of alpha and beta diversity measuring the diversity within the individual sample and the diversity between the samples has been conducted. The whole procedure is deciphered to result in a list of gut bacteria that could further be distributed in separate lists of phylum, class, order, family, genus, and species [13].

1.9.3 Microbiota-Gut-Brain-Axis in ASD Through Metabolites

Microbiota-Gut-Brain-Axis can be elucidated through the gut metabolites that have the potential to cross the BBB under the dysbiotic environment and affect the brain physiology. In the present study, metabolites produced in the gut by microbes have

been identified, and the connection of these metabolites with ASD has been explored. In order to achieve this objective, an in-silico methodology is developed to identify the metabolites produced by microbes residing in human gut.

The metabolites produced by various bacterial species have been identified through various online databases. The metabolic reactions of the identified metabolites have been carried out through Google Co-Lab and Metaboanalyst. All of the steps to achieve the objective have utilized databases and tools that are easily, freely, and publically available [14–16].

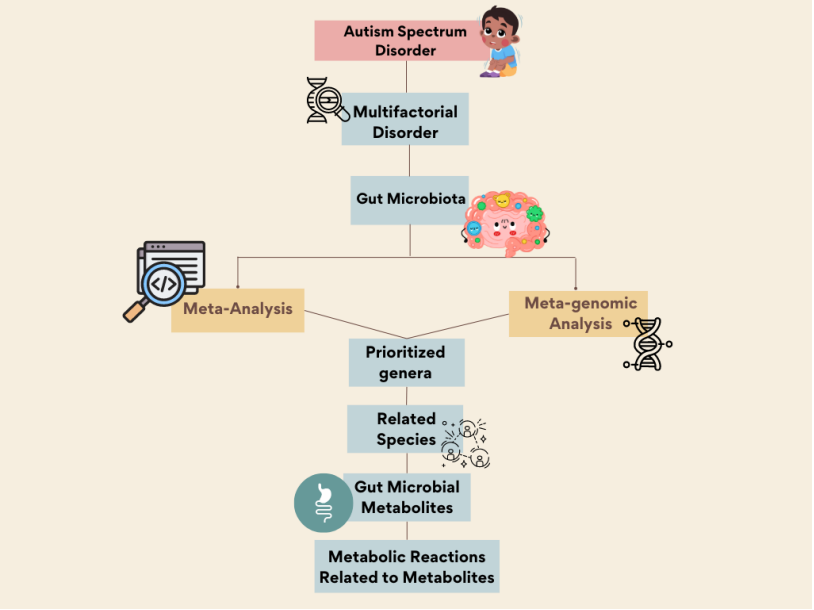

FIGURE 1.5: Depicts overall methodology employed in the current project.

Chapter 2

Literature Review

2.1 Autism Spectrum Disorder

Autism Spectrum Disorder is defined as a complex, heterogeneous, and multifactorial group of neurodevelopmental disorders manifesting short falls in three key domains including communication skills, social interactions, and repetitive activity, response or behavior [17]. Paul Eugen Bleuler, a Swiss psychiatrist initially used the word autism in 1912 to explain some signs of schizophrenia. In 1943, Leo Kanner used autism in the modern sense while describing 8 boys and 4 girls who could not establish effective eye contact with the others [18].

2.2 Prevalence of ASD

The prevalence of ASD has increased manifold over the last decade.

2.2.1 World-Wide Prevalence

Centre for Disease Control (CDC) reports that 27.6 per 1,000 children with the age of 8 years are being identified with ASD in United States, that is equivalent to 1 out

of 36 children aged 8 years. The disease also shows the gender biasness with the male to female prevalence ratio of 3.8.

The sharp rise in the prevalence of ASD lies on the comparatively better diagnosis opportunities and awareness among the masses. However, such sudden increase in the epidemiology intrigues the researchers to look deeper in to the etiology and pathophysiology of this disorder [2].

The world-wide data on prevalence is shown in figure 1, showing the estimates of prevalence from Asia, Europe, Africa, Australia, and America to be 0.4%, 0.5%, 1%, 1,7%, and 2.76% respectively [2, 19].

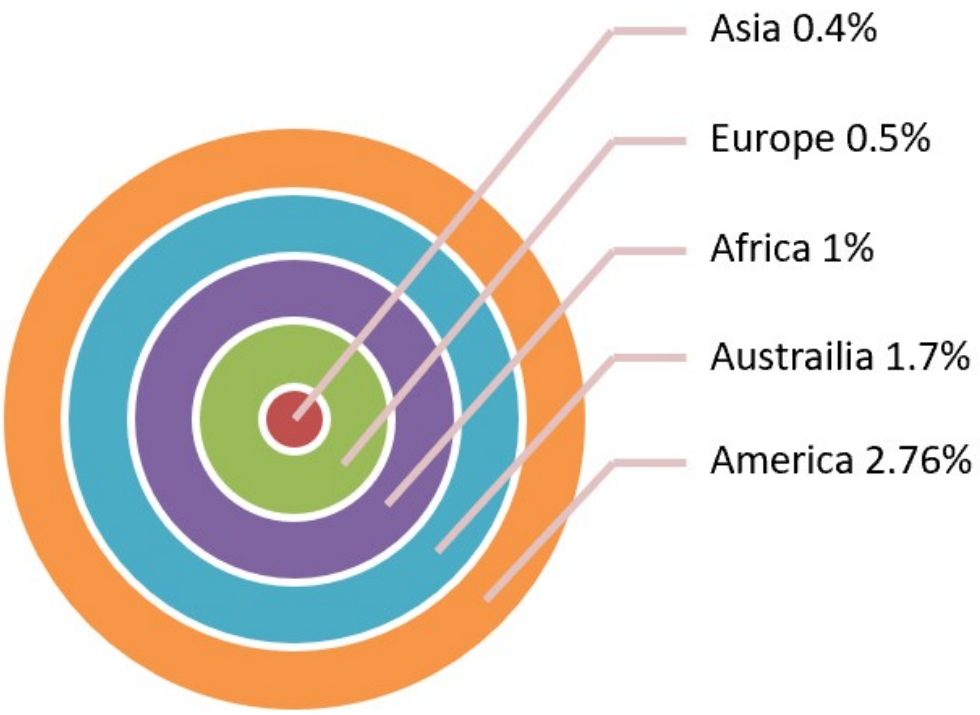

FIGURE 2.1: Global prevalence of ASD in recent past [2, 19].

2.2.1.1 Prevalence of ASD in Pakistan

There is a lack of data on Pakistani population that could help in assessing the prevalence of this disorder in Pakistan. However, in 2020, it was estimated by Pakistan Autism Society that 350,000 children in Pakistan are suffering from ASD [20–25].

2.3 ASD Symptomatology

The ASD patients find difficulties in communication and social interactions including inability to develop non-verbal interactions including facial expressions, eye contact, gestures and persistent deficits in social relationships like inability to share interests, and expression and understanding of emotions. These defects end up in deteriorated social relationships. They also exhibit restricted and repetitive behavior, activities or interests.

These unusual behaviors may exist in the context of stereotyped use of objects, language (idiosyncratic phrases, echolalia), and movements. A rigid constancy to routines, insistence on sameness, and extreme emotional outburst at small changes can also exist [21]. Some of the key symptoms affecting the major targeted domains in ASD are depicted in fig 2.2.

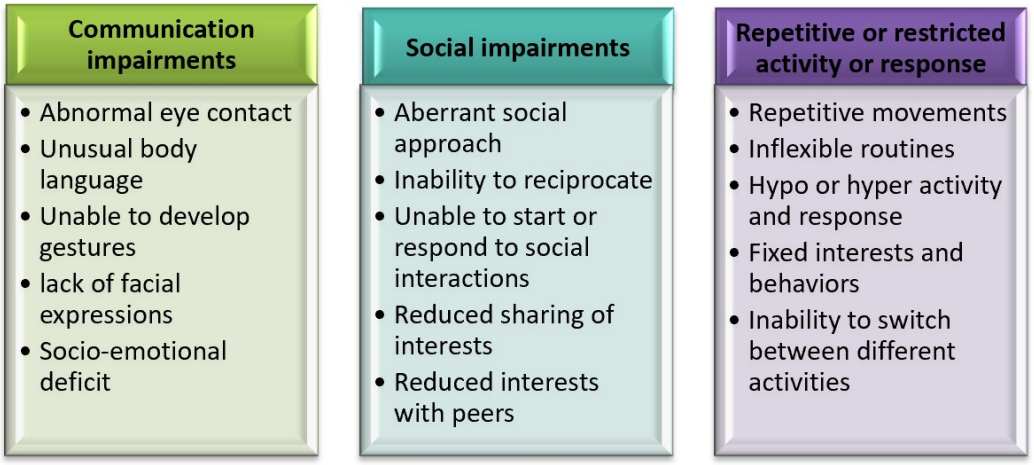

FIGURE 2.2: Key symptoms affecting three major domains in ASD.

2.4 Diagnosis of ASD

Diagnosis of ASD is purely done on behavioral bases, primarily due to the lack of early diagnostic markers. Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM-V) intends to diagnose ASD in a simple and straightforward manner.

DSM-V diagnoses a person with ASD having all issues in social interactions and communication domain such as in-sufficient social-emotional mutual exchanges, defective non-verbal cues for social communication, and an inability to develop, grow, and understand relationships, and two out of four short-falls in repetitive or restricted activity, behavior or response, including stereotypic motor movements, usage of things, or verbal communication, inflexibility in routines, abnormal restricted fixed interests and activities, and hypo or hyper activity to sensory stimulations.

The criteria introduces five specifiers for a better diagnosis of the disorder. The first and second specifier explains the presence or absence of intellectual impairment, language impairment respectively. The third, fourth, and fifth specifier depict the presence or absence of any known genetic, environmental, or medical condition, association to any behavioral, mental, or neurodevelopmental disorders, and catatonia respectively [17–23].

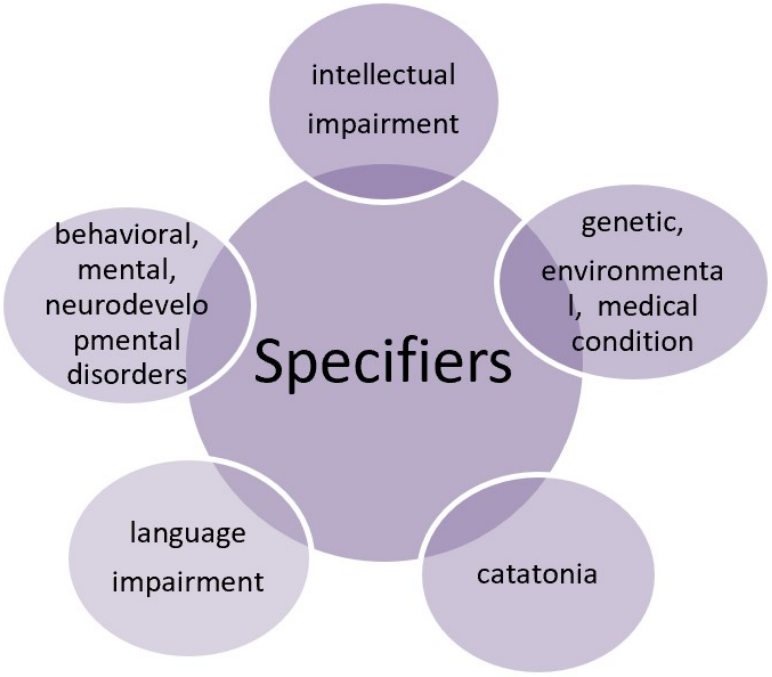

FIGURE 2.3: Different specifiers used for the diagnosis of ASD.

In addition to this, current severity specifiers that range from level 1 to 3, depending upon the need of support, substantial support, and a very substantial support are used for a better diagnosis of the disorder [21].

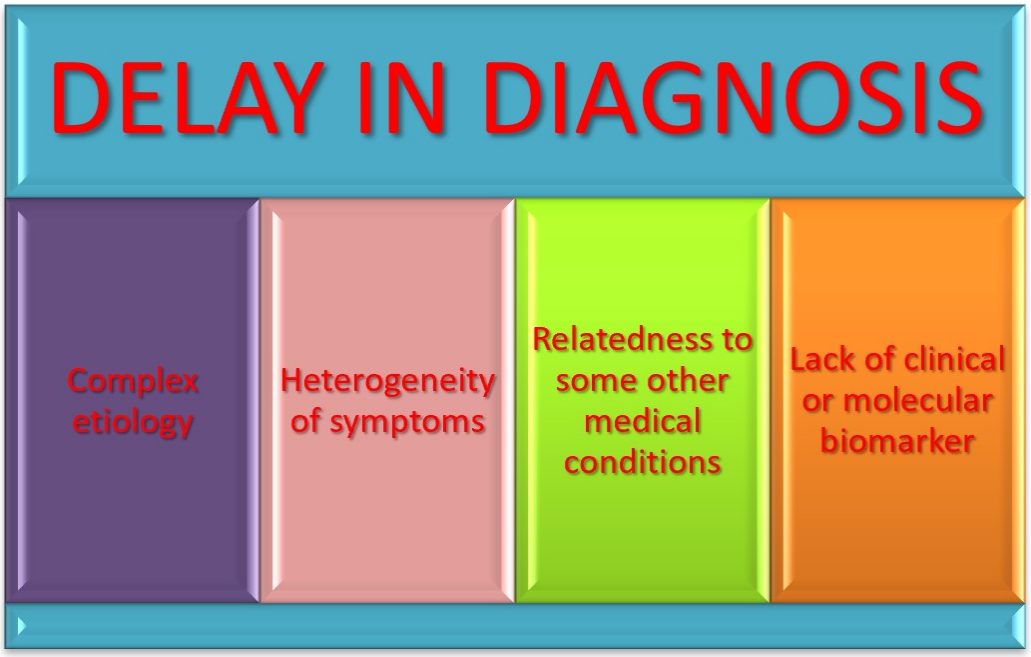

FIGURE 2.4: Various factors including obscure pathophysiological mechanisms, diversity in symptoms, and lack of biomarkers result in the delayed diagnosis of ASD.

The early diagnosis is very critical in intervention strategies and aids in reducing the social burden of autistic individuals. The ASD symptoms must be present during early developmental phases during childhood but may not be recognizable until the social demands exceed the limited capacities of the child to cope them and this is reason that autism is generally diagnosed in the 2-3 years of age and the heterogeneity of the symptoms also poses hindrance in diagnosis by the clinicians or the parents relate the symptoms to some specific behaviors not related to autism, and some other medical issues. Moreover, no clinical and molecular biomarkers are yet available that could aid in early diagnosis [23].

2.5 Spectrum Disorder

Autism is referred to as a spectrum disorder because the affected persons differ from one another with respect to the characteristics and severity of symptoms, including a wide range spectrum of phenotypes. The cognitive ability ranges from profound intellectual disability to above-average intellectual functioning, and language disability

sweeps through the complete absence of speech to the fluent language [24]. Several co-morbidities including epilepsy, intellectual disabilities, sleeplessness, sensory sensitivity, depression, immune disorders, and gastrointestinal disorders are also documented that ultimately ends in making the phenotype more complex and heterogeneous [25].

2.6 ASD as Financial Burden

ASD patients pose a serious economic burden on the family and society. The per capita costs of ASD is measured in some of the countries, and costs usually vary, primarily due to the difference in geographic regions, diagnostic opportunities, and awareness among the masses.

Recently, the economic cost of ASD is £ 2.3 billion in Scotland, $ 9645,503 in South Korea, and $ 41.8 billion in China [26–28]. Furthermore, the annual cost for ASD goes far to $ 268 billion in the US, which is further expected to rise by over $ 461 billion in 2025 [29].

2.7 Societal Pressures Associated with ASD

The co-morbid health issues, social and financial burden, and reduced acceptance from the society further worsens the situation. If ASD is not diagnosed well in time, it sweeps from childhood to the adulthood and persistently affects the personal, social, and professional life of the individual. The inability to carry on the basic daily life activities ranging from eating habits, reading, writing, learning, and social communication hampers the life of the individual [30, 31].

2.7.1 Family Life Disturbances

The effects of ASD on the society are not only limited to the social burden of autistic individual but also the menace of family life of siblings and parents. The parental

distress is evident in a recent study where families asserted that the autistic children negatively affected their family relationships and siblings' normal life along with deleterious effects on their own social lifestyles.

The painful element lies on the fact that these children are usually unable to express their sufferings, especially if they have language impairment that makes the parents and child even more aggressive because the child is unable to express his/her basic needs like hunger, thirst, sickness etc.

In some situations, parents have to socially isolate themselves because they are unable to expose their child to the community who may not understand the actual condition and might hurt the feelings of the parents as well as the child [31].

Moreover, a recent study in Saudi Arabia has shown that societal pressures, reduced quality of life, depression, economic pressures, feelings of uneasiness, and discomfort are common among the caregivers and parents of autistic children, that affects the quality of care and support offered to autistic children, and ultimately worsens the symptoms of ASD [32].

2.8 Pathophysiology of ASD

The underpinnings of the ASD etiology are complex and obscure, making it a challenge for the scientific community.

2.8.1 Earlier Psychiatric View

Early infantile autism initially described by Kanner was identified as a severe psychiatric or behavioral disorder that was diagnosed in early infancy. Kanner was also a proponent of the psychogenic approach and claimed that the main cause of autism was the lack of parental care and love, especially the lack of warmth by the mothers, and the cold attitude of parents. However, later research disproved this theory and highlighted the neurobiological and genetic factors contributing to autism [18].

2.8.2 Psychiatric to Biological Shift

Kanner idea of parents to be responsible for autism had been controversial and with the advent of modern research, a shift from emotional and psychogenic causes to the biological ones became dominating and autism started emerging as a neurological disorder. Thus a major change in the notion of ASD was made forward by moving from psychogenic origins towards the biological origins [33].

The underlying mechanisms delineating the pathophysiology of the ASD include aberrations in the assembly or structure of transmembrane, synaptic cell adhesion, and scaffolding proteins that are primarily involved in the development and maintenance of synapses, as well as the dysfunctions in cellular signaling pathways that play the critical functions to control synaptogenesis and axon motility [34].

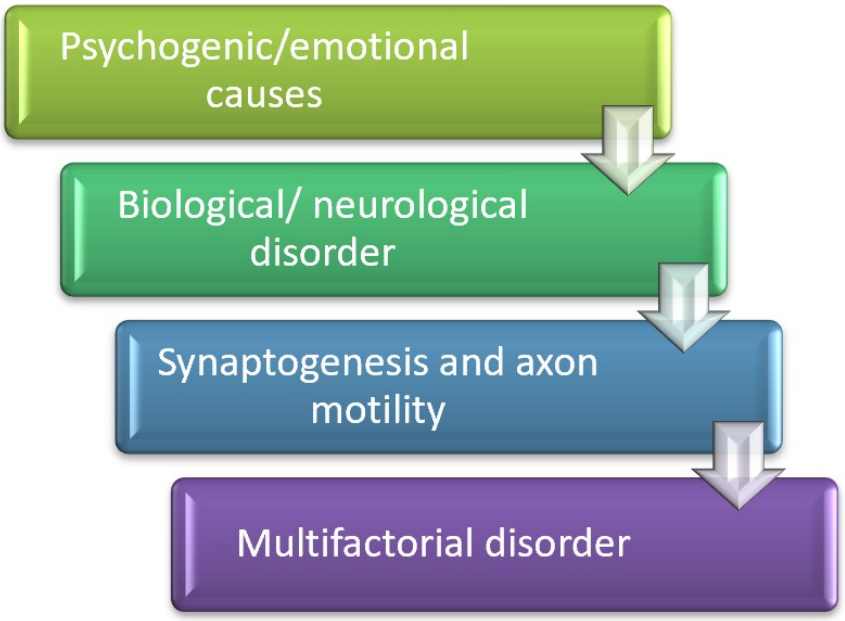

FIGURE 2.5: Multiple approaches towards the pathophysiology of ASD.

2.8.3 Multifactorial Disorder

ASD has emerged as a multifactorial disorder with various risk factors ranging from genetics, epigenetic, and environmental factors.

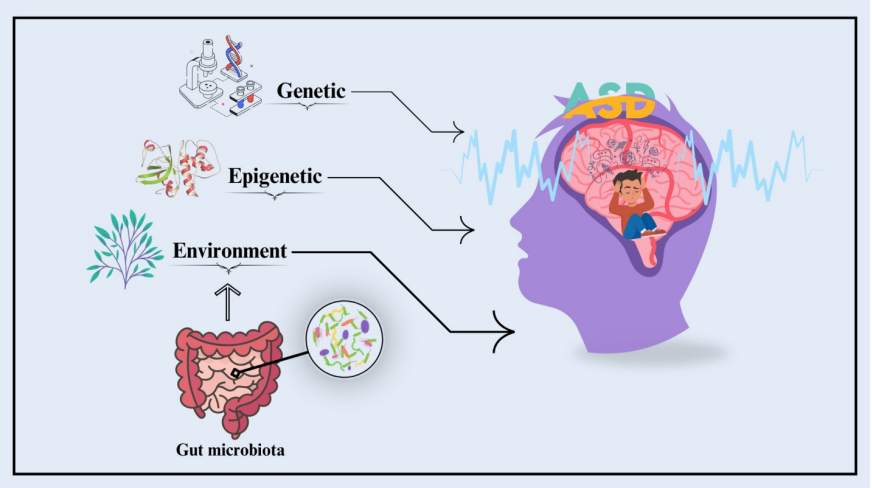

FIGURE 2.6: Multiple contributing factors in the pathyphysiology of ASD.

2.8.3.1 Genetic Bases

A genetic basis has been proposed and various ASD cases have been linked to genetic causes including genetic defects, chromosomal anomalies, and associated syndromes. Though over 800 genes have been strongly linked to the risk, but no "autism gene" has yet been identified that could strengthen the impact of genetics in the etiology [35]. Several chromosomal aberrations and single gene mutations are linked with ASD. Some of the relevant candidate genes are shown in table 2.1 [36].

TABLE 2.1: Candidate genes associated with ASD

Sr. No.	Name of Gene	Gene Symbol
1	Ankyrin repeat domain 11	ANKRD11
2	AT-rich interaction domain 1B	ARID1B
3	ASXL Transcriptional Regulator 3	ASXL3
4	ATRX Chromatin Remodeler	ATRX
5	Autism susceptibility candidate 2	AUTS2
6	Chromodomain helicase DNA binding protein 2	CHD2
7	Chromodomain helicase DNA binding protein 7	CHD7
8	Chromodomain helicase DNA binding protein 8	CHD8
9	CREB-binding protein	CREBBP

10	Euchromatic histone-lysine N-methyltransferase 1	EHMT1
11	Methyl-CpG binding domain protein 5	MBD5
12	Methyl CpG binding protein 2	MECP2
13	SET domain containing 5	SETD5
14	Activity-dependent neuroprotector homeobox	ADNP
15	Forkhead box G1	FOXG1
16	Forkhead box P1	FOXP1
17	Forkhead box P2	FOXP2
18	Mediator complex subunit 13-like	MED13L
19	Pogo transposable element with ZNF domain	POGZ
20	Retinoic Acid Induced 1	RAI1
21	T-box, brain 1	TBR1
22	Transcription factor 4	TCF4
23	Zinc finger and BTB domain containing 20	ZBTB20
24	Fragile X mental retardation 1	FMR1
25	Ubiquitin protein ligase E3A	UBE3A
26	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	DYRK1A
27	Neurofibromin 1	NF1
28	Phosphatase and tensin homolog	PTEN
29	Synaptic Ras GTPase activating protein 1	SYNGAP1
30	Tuberous sclerosis 1/2	TSC1/TSC2
31	Cyclin-dependent kinase-like 5	CDKL5

2.8.3.2 Epigenetic Mechanisms

Epigenetics refers to the mechanisms that can result in changes in gene expression without any alteration in gene sequences. Such mechanisms include genomic imprinting, epimutations, DNA methylation, histone modification, and microRNA. They alter

gene expression without modifying the genetic sequence and are linked to several neurodevelopmental disorders, including ASD. These epigenetic modifications can be influenced by environmental factors such as diet, stress, and exposure to toxins. Studies suggest that abnormal epigenetic regulation may contribute to the onset and progression of ASD by affecting critical genes involved in brain development and neuronal function. Understanding these mechanisms can help develop potential therapeutic strategies for ASD and other neurodevelopmental disorders [37].

2.8.3.3 Environmental Factors

More recently, environmental factors including prenatal viral infection, maternal diabetes, certain toxins, heavy metals, environmental insecticides and pesticides, immunological proteins, food contaminants, parental age, maternal smoking and alcohol consumption, and gut microbiota have emerged as the potential risk factors for ASD [37, 38]. The genetically susceptible patterns may become target of environmental threat and result in dysregulations of neurodevelopmental pathways, but these complex interactions are difficult to be identified due to varied environmental factors that might be playing their roles [39]. A possible way to tackle these diverse problems could be to consider highly associated comorbid conditions such as Gastro Intestinal Symptoms (GIS) along with the typical symptoms of the pathology.

2.8.3.4 Gastro Intestinal Symptoms

Gastro Intestinal Symptoms (GIS) are a common co-morbidity in autistic children. These GIS are identified as five times more common in ASD subjects, with constipation and diarrhea four times more prevalent, and abdominal pain as two times, when compared to children without ASD [39, 40]. The frequent GIS identified in ASD subjects include diarrhea, flatulence, burping, bloating, constipation, and abdominal pain. The prevalence range for GIS for ASD variates from 9% to 84% as compared to 9% to 37% in neurotypically (NT) developing children [41]. Moreover, the severity of ASD behavior is strongly linked to the prevalence of these GIS. Children with ASD

exhibit more aggression, tantrums, sleep disturbances, self-injury, anxiety, and anger, which can partly be due to the physical disturbances caused by GI disturbances [42]. Thus early diagnosis and treatment of these GIS might prove helpful to reduce or remove some of the above mentioned behavioral problems. It is speculated the GIS appear as a phenotypic manifestation of a condition involved in the pathophysiology of this complex disorder, or at least in some of the subgroups. Gut microbiota might be a promising subject for this underlying condition.

2.9 Treatment Approaches

Treatment and prevention approaches for ASD rely to focus on the symptoms and the co-morbid conditions, rather than treating the core ASD symptoms as no standard treatment protocol for ASD has yet been established.

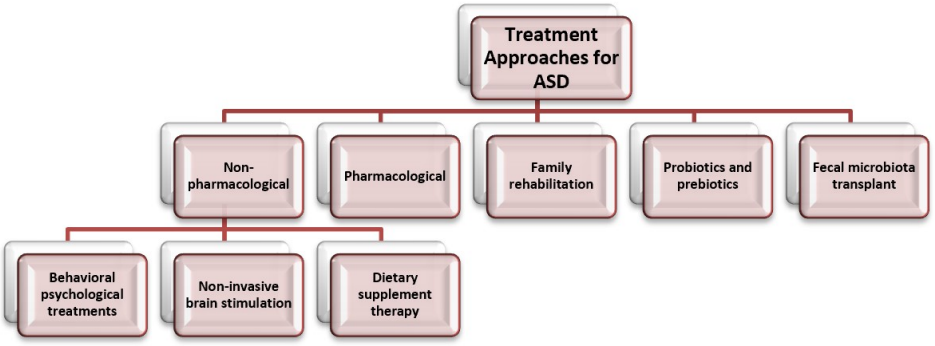

FIGURE 2.7: Treatment approaches used for ASD.

Non-pharmacological therapies, pharmacological protocols, family rehabilitation, and use of probiotics, prebiotics, and fecal microbiota transplant are in use to alleviate the spectrum of phenotypes in ASD [43].

2.9.1 Non-Pharmacological Therapies

Various Non-pharmacological therapies including behavioral psychological treatments, brain stimulation, and dietary supplementation therapy is offered to ASD individuals.

Applied Behavior Analysis (ABA), language and speech therapies, and physical therapy services, music treatments, cognitive behavioral therapy, and social behavioral therapies are offered for management of the disorder.

These treatments have some positive effects on ASD like improvement in anxiety and depression, as well as develop functional independence among ASD individuals [17]. In addition to it, certain non-invasive brain stimulation procedures like transcriptional magnetic stimulation and transcriptional direct current stimulation are used. Both of these procedures have resulted in improved social behavior and cognition [44].

Several lines of study report that certain vitamins such as vitamin D, B6, B12, folic acid, and omega-3 polyunsaturated fatty acids have shown to improve the ASD co-morbidities like GIS, and dysbiosis caused due to gut microbes, improve overall gut health, and reduce the behavioral symptoms [45].

2.9.2 Pharmacological Protocols

Pharmacological protocols are employed in order to manage the symptoms and co-morbidities that are linked with ASD. These drugs include some anti-psychotic medications, psychostimulants, anti-depressants, and mood stabilizers. These medicines aid in managing some ASD symptoms like anxiety, depression, agitation, behavioral problems, and gastrointestinal dysfunction but some side effects are also associated with them. Moreover, there is no drug that has yet been established for the core symptoms of ASD [46].

Table 2.2: Drugs used for symptoms and co-morbidities in ASD.

Sr. No.	**Drugs**	**Category**	**Targeted Symptoms**	**Side Effects**	**Ref.**
1	Aripiprazole	Atypical antipsychotic	Irritability, self-aggression, repetitive behavior	Sedation, quivering, drooling, weight gain	[17]

2	Risperidone	Atypical antipsychotic	Aberrant social behavior, delayed developmental phases, self-aggression	Weight gain	[43]
3	Guanfacine	Alpha-2-adrenergic Agonists	Attention Deficit Hyperactivity Disorder (ADHD), disuptive behavior	Sedation, tiresome, reduced blood pressure and pulse	[44]
4	Methylphenidate	Stimulant	Repetitive behaviors, hyperactivity	Reduced appetite, sleep disturbances	[45]
5	Atomoxetine	Noradrenaline reuptake inhibitor	ADHD, Repetitive behaviors	Decreased appetite, irritability, nausea	[45] [46]
6	Melatonin	Antipsychotic	Pain, anxiety, depression, gastrointestinal dysfunction	Headaches, dizziness	[47]

2.9.3 Family Rehabilitation

Family rehabilitation focuses around the psychological approaches towards the family members to better understand the communications and interactions within a family. This therapy aims to provide basic knowledge about ASD, its causes, diagnostic approaches, preventive measures, and coping with the core as well as the associated symptoms and co-morbidities of ASD. It also helps in fostering a supportive environment, enhancing parental coping strategies, and promoting the social and emotional development of children with ASD. By strengthening family dynamics, this approach

contributes to improving the overall quality of life for both the child and their caregivers. [46].

2.9.4 Probiotics, Prebiotics, and the Fecal Transplant

Recently, the use of probiotics, prebiotics and the fecal microbiota transplant is in trial to alleviate the symptoms of gastrointestinal troubles. Prebiotics and probiotics have shown to reduce the degree of ASD severity, and GI related dysfunctions. The exact mechanism of action of these is unknown, yet it is speculated that the healthy effects on the integrity of gut barrier, and ability to manipulate the composition and functioning of gut bacteria ameliorates the symptoms associated with ASD [48]. Positive effects on sleep disturbances, GI symptoms, improved gut bacterial diversity maintaining healthy gut environment are observed by giving fecal microbiota transplant [49].

Though no standard treatment to ASD exists except some behavior therapies, earlier detection could aid in offering management therapies to alleviate the symptoms and uplift the quality of life in autistic individuals. Early detection, understanding pathophysiology, and developing treatment protocols are crucial to improving life quality and reducing societal burden. However, ASD's etiology remains complex, involving genetic, epigenetic, and environmental factors. The interplay between these factors is speculated in its pathophysiology. Notably, around 90% of ASD cases exhibit gastrointestinal symptoms (GIS), which correlate with autism severity and can be alleviated by prebiotics and fecal microbiota transplants. This highlights gut microbiota as a promising target for further exploration to uncover ASD's obscure etiology.

2.10 Gut Microbiota

The microbes such as fungi, bacteria, viruses, protozoa, bacteriophages, and archaea residing in the GI tract are referred to as gut microbiota. The term microbiota generally refers to the bacteria alone in spite of the presence of other microbes owing to the overwhelmingly greater number of bacteria as compared to other microbes.

More than 1014 bacteria inhabit the human GI tract, and their genome is about 100 times bigger as compared to the genome of human [50].

2.10.1 Importance of Gut Microbiota

Gut microbiota establish a symbiotic relationship with human GI tract, and perform certain important roles in the human body including neurological and endocrine functions, metabolization of in-digestible carbohydrates, controlling immune system, producing necessary vitamins and antimicrobial substances, providing shelter against infective pathogens, and production of mucine [8].

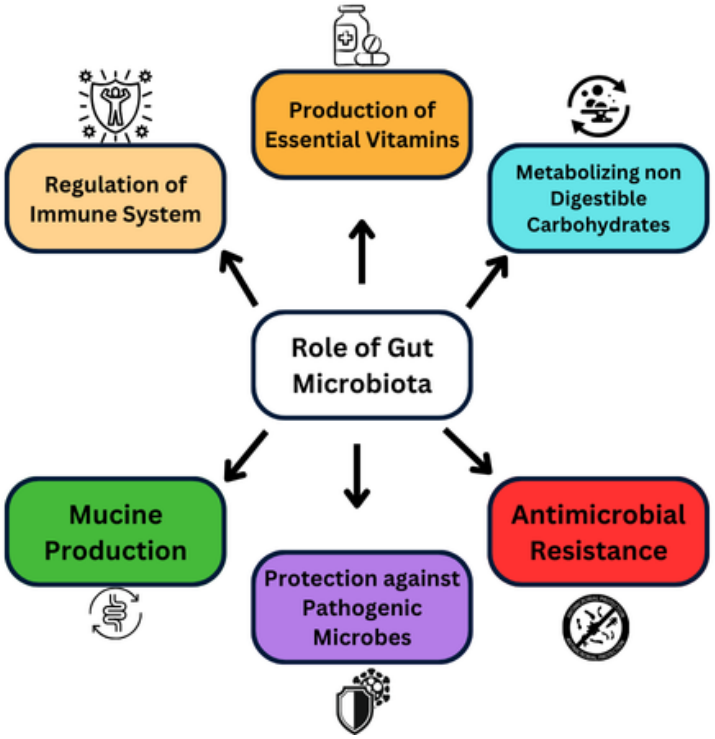

FIGURE 2.8: Functions performed by gut microbiota in human body.

2.10.2 Importance of Gut Microbiome in Relation to Health and Immunology

The key domains of the human body whose functions are regulated by the GM include structural, protective, metabolic, and neurological.

2.10.2.1 Metabolic

Dietary Fibers

The human gut is capable of digesting about 85% of the taken carbohydrates, 66-95% of the proteins, and almost all of the fats. However, the constraint of the human digestive system is to digest certain crucial dietary fibers such as oligosaccharides, non-starch polysaccharides, resistant starch, and lignin. Gut microbiota helps the human digestive system to digest such resistant carbohydrates by the virtue of certain glycosidase enzymes like polysaccharide lyases, carbohydrate es-

terases, glycoside hydrolases, and glycosyltransferases [1]. The fermentation of such complex dietary fibers by GM results in the release of certain gases such as carbon dioxide, hydrogen, and methane. In addition to it, certain organic acids like succinate and lactate, alcohols like methanol, and ethanol, and certain SCFAs including acetate, formate, butyrate, and propionate are produced by the fermentation process. Peripheral tissues utilize acetate as an energy substrate. Moreover, the key processes in liver such as cholesterol biosynthesis, and lipogenesis are controlled by acetate [2].

Butyrate, primarily utilized by colonocytes, functions as an energy source and is also involved in the production of ketone bodies and carbon dioxide. Apart from serving as an energy source, butyrate plays a crucial role in maintaining energy balance by activating enteroendocrine cells in human gut, which stimulates the release of leptin from fat cells and enhances the formation of glucagon-like peptide-1 (GLP-1) in L cells. Furthermore, butyrate assists in reducing the impact of toxic metabolites, such as bile acids and phenolic compounds. Propionate, on the other hand, is taken up by colonocytes and carried to the liver, where it performs functions comparable to those of acetate [3].

2.10.2.2 Protective

The gastrointestinal tract acts as a vital connection between the immune system and the GM. The initial layer of the gut immune system, which includes gut-associated

lymphoid tissue and Peyer's patches, forms through interactions with gut commensals. This immune barrier restricts direct contact between commensals and epithelial cells. The second immune layer quickly detects and eliminates bacteria without tissue invasion. A tertiary layer of immune responses operates locally within the mucosal tissue, engaging mucosal immunity without triggering systemic immune activation. The innate immune defense comprises of mucus, antimicrobial peptides, and secretory immunoglobulin A (IgA) [4].

Innate and Adaptive Immunity:

Commensal microorganisms are distinguished from pathogens by the virtue of innate immunity, primarily via germline-encoded pattern recognition receptors, including Toll-like receptors (TLRs). Some preserved pathogen associated molecular patterns such as lipoproteins, unmethylated CpG DNA, flagellin, and bacterial lipopolysaccharide (LPS) are recognized by TLRs. Lipids are identified by TLR1, TLR2, as well as TLR6. TLR5 recognizes flagellin, while TLR4 interacts with a range of ligands, including lipopolysaccharide (LPS) and paclitaxel.

Nucleic acids are identified by TLR7, TLR8, and TLR9 [5]. NOD-like receptors recognize cytosolic bacterial antigens. Antigen-presenting cells (APCs) which include macrophages and dendritic cells are known to express the TLRs, phagocytose pathogens, and secrete cytokines to activate naïve CD4+ T cells, differentiating them into Treg, TR1 subsets, TH1, TH2, and TH17.

A critical balance between effector T lymphocytes and Treg cells in human gut helps distinguish between commensals and pathogens. Dysbiosis can trigger inflammatory responses through TH1, TH2, as well as TH17 cells, with pro-inflammatory cytokines like IFN-γ, IL-6, IL-4, IL-13, IL-5, IL-22, IL-17, and IL-8 [6].

Microbial Metabolites in Immunity

Undigested carbohydrates that are not absorbed in the small intestine are fermented by microbiota that reside in large intestine, undergoing saccharolytic fermentation to generate short-chain fatty acids (SCFAs), such as butyrate, acetate, and propionate.

Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are absorbed by host cells and interact with G protein-coupled receptors (GPCRs) such as GPR43, GPR41, and GPR109A that are present on epithelial and immune cells [2]. Butyrate specifically links to GPR43, triggering the formation of certain anti-inflammatory cytokines such as IL-10 and TGF-β, while also upregulating FOXP3 expression in Treg cells. Additionally, histone deacetylase activity is inhibited by butyrate, and it also attenuates NF-κB-driven inflammatory responses. Studies indicate that acetate has a higher affinity for GPR43 as compared to butyrate. Acetate administration enhances IgA production in wild-type mice, an effect not observed in mice where GPR43 has been knocked out. Furthermore, acetate promotes the expression of Aldh1a2 inside the dendritic cells, facilitating the conversion of vitamin A to retinoic acid, which subsequently stimulates mucin and IgA secretion by B cells and goblet cells, thus reinforcing the intestinal barrier [7]. Mice deficient in GPR43 exhibit heightened vulnerability to dextran sodium sulfate-induced colitis, with increased neutrophil chemotaxis and elevated inflammatory gene expression. Co-treatment with propionate and butyrate attenuates the inflammation induced by LPS, by the activation of Treg cells and reducing the secretion of pro-inflammatory cytokines such as IL-12 and IL-6 [8].

2.10.2.3 Structural

The intestinal epithelium is composed of a monolayer of columnar cells, closely bound by junctional complexes that control the permeability of the paracellular space. These complexes include tight junctions such as zonula occludens, adheren junctions called as zonula adherens, and desmosomes. Beneath the tight junctions, there exist the adherens junctions, and they constitute apical junctional complexes that are linked to the actin cytoskeleton. The actin filaments bridge these complexes, facilitating cell-cell adhesion and intracellular signaling, while the adhesive forces that are crucial for the integrity of intercellular interactions are provided by adherens junctions and desmosomes [9]. The tight junctions create a selective barrier with size and charge selectivity, comprising two distinct pathways: the 'pore' pathway, which is high-capacity,

size- and charge-selective, and the 'leak' pathway, which is low-capacity and less selective [4]. Enterotoxins from pathogens like enteropathogenic C. perfringens, C. difficile, and E. coli can compromise tight junction integrity, weakening the barrier function. Cytokines such as IL-13, IL-6, IL-4, IL-1β, TNF-α, and IFN-γ promote the disruption of tight junctions, increasing intestinal permeability. In contrast, cytokines like IL-10, IL-17, and TGF-β help restore the intestinal barrier, reducing permeability [10]. Nutrients and dietary components, including glutamine deficiency, ethanol, acetaldehyde, and fatty acids can enhance intestinal permeability. In contrast, amino acids like tryptophan and glutamine, casein-derived peptides, short-chain fatty acids (SCFAs), vitamins A and D, and polyphenols help to mitigate luminal content penetration. Various nutrients and diet related factors like glutamine deprivation, fatty acids, ethanol, and acetaldehyde can increase permeability, while amino acids like glutamine and tryptophan, along with casein peptides, SCFAs, vitamins A and D, and polyphenols reduce luminal content infiltration [11].

2.10.2.4 Neurological

The two-way signaling that exists between the gastrointestinal system and the brain, known as the gut-brain axis (GBA), is mediated through the enteric nervous system (ENS), which interacts with the sympathetic nervous systems, the parasympathetic nervous systems, and the hypothalamic-pituitary-adrenal (HPA) axis [12]. The enteric nervous system (ENS), frequently referred to as the "second brain," consists of trillions of neurons and is structured into two main plexuses: the myenteric and submucosal ganglia. GM can influence ENS and as well as the central nervous system (CNS) via several mechanisms, including

1. the synthesis, expression, and turnover of neurotransmitters and neurotrophic factors,
2. maintenance of intestinal barrier function and tight junction integrity,
3. modulation of enteric sensory afferents,

4. production of bacterial metabolites, and

5. regulation of mucosal immunity.

The enteric nervous system is capable of detecting various neurotransmitters, many of which are also present in the CNS, such as serotonin, acetylcholine, and dopamine. Notably, major bulk of serotonin (approximately 90%) and almost 50% of dopamine are synthesized in the gut, primarily by gut microbiota. These neurotransmitters are essential for transmitting "fight or flight" signals to the brain and regulating mood, pleasure, and happiness [13]. Bacterial metabolites, particularly short-chain fatty acids (SCFAs), stimulate the expression of tryptophan hydroxylase 1 in enterochromaffin cells, thereby increasing serotonin secretion in the gastrointestinal tract. This serotonin, in turn, can activate the sympathetic nervous system, influencing cognitive processes like memory and learning.

Dysbiosis that occurs in gut microbiota, particularly in functional gastrointestinal (GI) disorders, has been linked to disruptions in the GBA, potentially contributing to mood disorders. Experimental research indicates that probiotics may enhance the levels of brain-derived neurotrophic factor (BDNF) in the cerebral cortex and hippocampus, thereby improving cognitive function and promoting muscle repair, regeneration, and differentiation [14].

2.11 Gut Microbiota Modulating Epigenetics

As a crucial symbiotic partner of the human body, microbiota can influence the host's epigenetic landscape. The human body can respond to environmental cues through various epigenetic regulatory mechanisms such as histone modifications, and DNA methylation [1]. Gut microbiota influence host epigenetic regulation, mainly through the production of metabolites that help preserve the body's dynamic equilibrium.

For example, the production of short-chain fatty acids (SCFAs) can alter the host's epigenome, subsequently impacting the organism's health status and susceptibility to

various diseases [2]. Gut microbiota is capable of synthesizing bioactive compounds that serve as precursors, such as methyl or acetyl groups, which are involved in histone modification and DNA methylation.

These compounds can influence the physiological and pathological mechanisms underlying host epigenetic regulation [3]. Gut microbiota is connected to host epigenetic regulation through the production of biomolecules derived from the metabolism of the host's diet. These include short-chain fatty acids (SCFAs), vitamins, polyamines, polyphenolic compounds, and tryptophan metabolites [4]. The swift fluctuations in short-chain fatty acids (SCFAs) triggered by variations in dietary nutrients or environmental factors can lead to subsequent epigenetic alterations in the host. For instance, butyrate plays a role in stimulating intestinal cell proliferation and sustaining homeostasis, acting through multiple signaling pathways [5].

The gut microbiota as well as the metabolites produced by the microbiota can drive distinct epigenetic alterations to modulate various physiological processes of the host. For example, short-chain fatty acids (SCFAs) generated through microbial metabolism serve as key energy sources for both the host's intestinal epithelial cells and the gut microbiota [6]. A key role of short-chain fatty acids (SCFAs) is to maintain the host's homeostasis by modulating epigenetic mechanisms. The predominant SCFAs in the colon are propionate and acetate. Propionate is primarily generated via the succinate pathway by Bacteroides, Roseburia species, Veillonella species, and Ruminococcus [7]. Propionate and butyrate can suppress the activity of histone deacetylases (HDACs) in intestinal epithelial cells (IECs) and immune cells by enhancing the hyperacetylation of histones and specific transcription factors involved in signal transduction. As a result, they play a crucial role in the progression of cancer [8].

2.11.1 Associations between Gut Microbiota and ASD

The connection between ASD and gut microbiota is also speculated on the stance that microbial composition stabilizes between 6 to 36 months of age, and this time is also critical for central nervous system development as synapse formation, maturation,

and myelination becomes more extensive at this window of time. Moreover, the gut microbial composition show variation in autistic children as compared to normal ones, and this microbial derangement is also linked to the severity of GIS in ASD patients. This opens up new avenues in ASD research by exploring gut microbiota and linking their association with ASD [51].

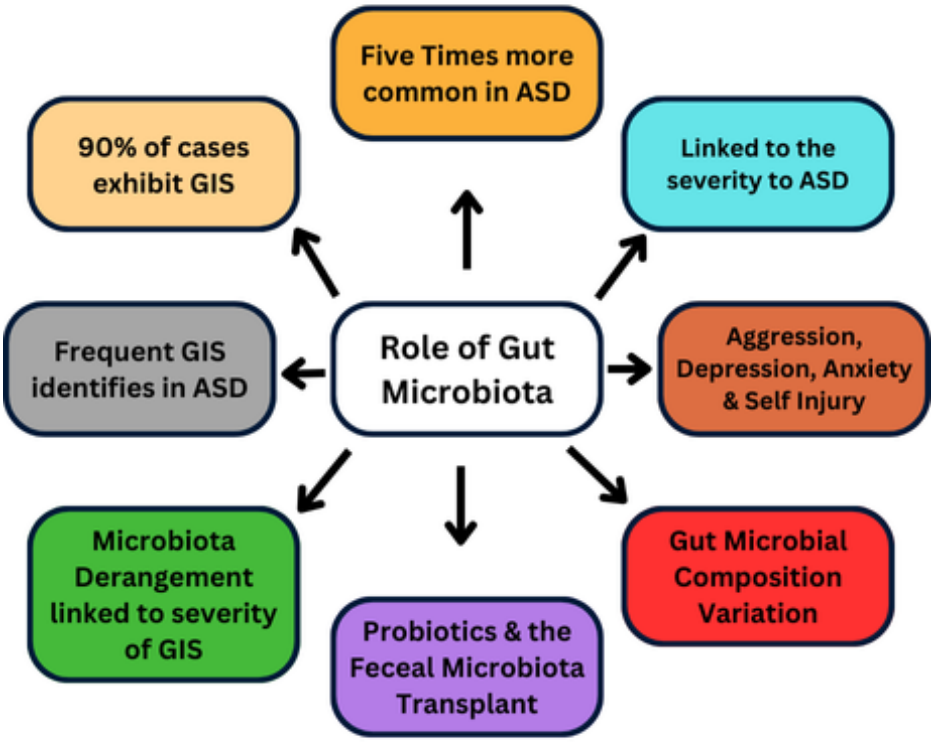

FIGURE 2.9: Various associations between gut microbiota and ASD strengthen the role of gut microbiota in ASD.

2.11.2 Dysbiosis in Human Gut

The dominant gut bacterial phyla in human body include Fermicutes, Bacteriodetes, Actinobacteria, Proteobacteria, and Verrucomicrobia, constituting about 97.2% of the total microbes residing in the gut, with Fermicutes and Bacteriodetes as the most dominating ones [8, 12]. Dysbiosis is the condition where the composition of gut microbes is changed, and resultantly the growth of pathogenic microbes is favored

over those having useful characteristics, thus compromising the health of the individual [52].

2.11.2.1 Reasons for Dysbiosis

The underlying reasons contributing to the altered microbial composition and leading to dysbiosis include maternal factors like maternal diet, maternal obesity, gestational diabetes, hospitalization, maternal infection, preterm babies, mode of delivery, and postnatal factors such as feeding pattern, antibiotics administration, and host genetics [46]. Maternal high fat diet during gestation decreases the level of Bacteriodes, and has been associated with dysbiosis and autism like behavior in mice [53]. Preterm babies have more pathogenic microbes surviving in their stomach and show abundance of Proteobacteria and genera like *Bifidobacterium* and Lactobacillus are found lesser in number [54].

With reference to mode of delivery, vaginally born babies have the healthiest microbial composition having abundance in Lactobacillus, Prevotella, Sneathia, and *Bifidobacterium* and decreased *Clostridium* deficile [55] while caesarean section delivered babies exhibit an altered microbial composition that resembles the skin of mother, having more abundance of Staphylococcus, Corynebacterium, and Propionobacterium [56]. Feeding pattern of the neonates also affect the microbial composition. Formula fed infants have more abundance of *Clostridium* deficile and liable to ASD, whereas breast-fed babies are lesser prone to autism [57].

Antibiotics administered during the first 3 years of life have disastrous effects on the healthy microbial composition. The antibiotics disrupt the colonization of the *Bifidobacterium*, a healthy microbe [58]. Microbial composition also shows variations if mother suffers from infection during prenatal time period. The infection leads to increased level of cytokines that are pro-inflammatory in nature in the maternal blood like IL-6, which performs numerous functions like regulation of tight junction proteins in terms of their expression, regulates synaptic plasticity, cognition, and neurodevelopment [59].

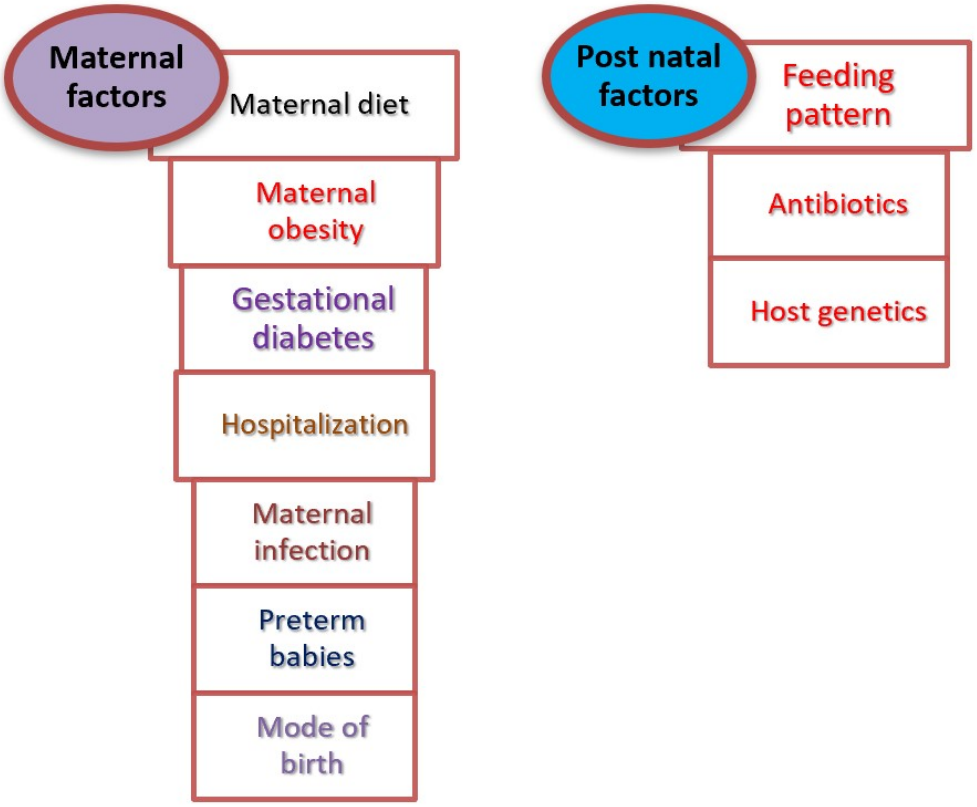

FIGURE 2.10: Illustration of various maternal, pre natal and post natal factors involved in dysbiosis.

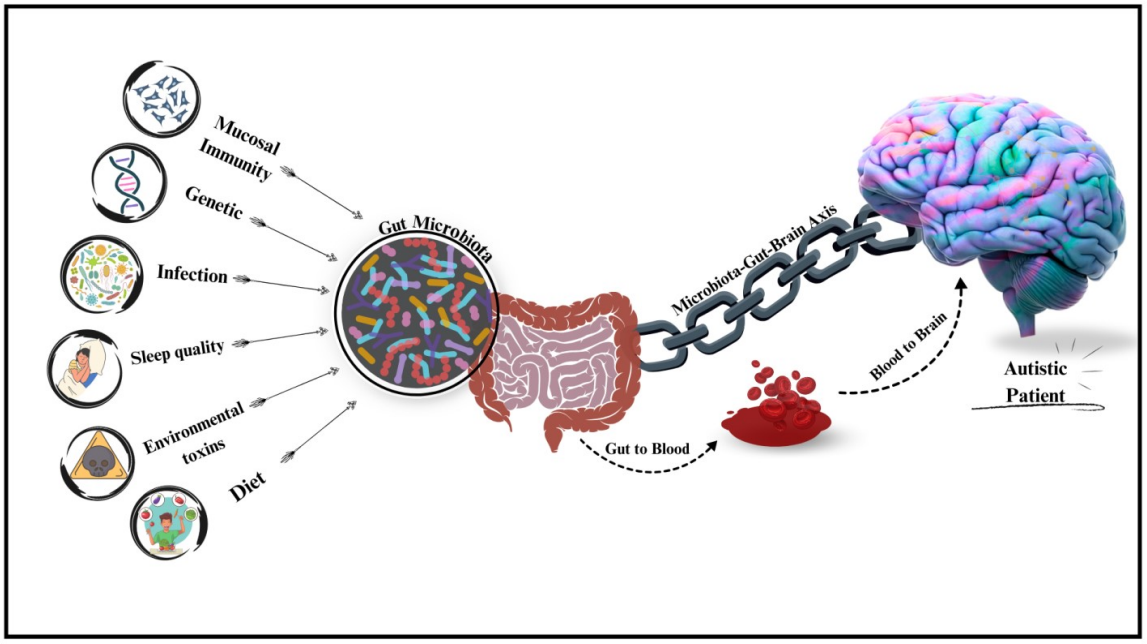

FIGURE 2.11: Various factors affecting the gut microbiota leading to dysbiosis and ultimately disturbing microbiota-gut-brain axis.

2.11.3 Microbiota-Gut-Brain Axis

The microbiota-gut-brain axis is the route of exchange of information that occurs between the gut microbes, and brain. The variations in gut microbiota has drastic effect on the brain development, and physiology through the bidirectional communication pathways between the gut microbiota and brain called the microbiota-gut-brain axis. How the gut microbiota affects the brain physiology is an important perspective in the exploration of pathophysiology of the ASD [8, 60].

2.11.3.1 Bi-directional Communication Pathways

The Neuroendocrine, autonomic nervous, toxins production, immunological and meta -bolic system pathways are the proposed two-way cross-talk pathways between gut microbes and central nervous system [61]. The increased permeability of gut barrier paths has been proposed to significantly affect the CNS in bidirectional interaction pathway.

The GI barrier comprising of commensal gut microbiota, epithelial cells, and a mucus layer connected through tight junctions becomes defective due to increased permeability of the intestine (referred to as 'leaky gut') in the ASD cases, delineating the relationship between ASD and the gut, as the bacterial metabolites and toxins enter in to the bloodstream, cross the blood brain barrier and trigger the immune response by releasing inflammatory cytokines, affecting the brain function [62].

The gut microbiota has been shown to regulate the intestinal permeability. The lower levels of intestinal tight junction components (CLDN-1, OCLN, TRIC) and higher levels of pore-forming proteins, claudin (CLDN)-5, CLDN-12, CLDN-3, and MMP-9) in ASD individuals as opposed to the controls have been evidenced [63].

Neuroanatomical Pathways

Neuroanatomical pathways including enteric nervous system, and autonomic nervous system and the vagus nerve which carry the signals from the human intestine to the

CNS [64]. Certain anatomical differences including increased expression of microglial cells and reduced number of purkinje cells have been observed in the brains of autistic individuals. The decreased number of purkinje cells results in impaired GABAergic functioning [65].

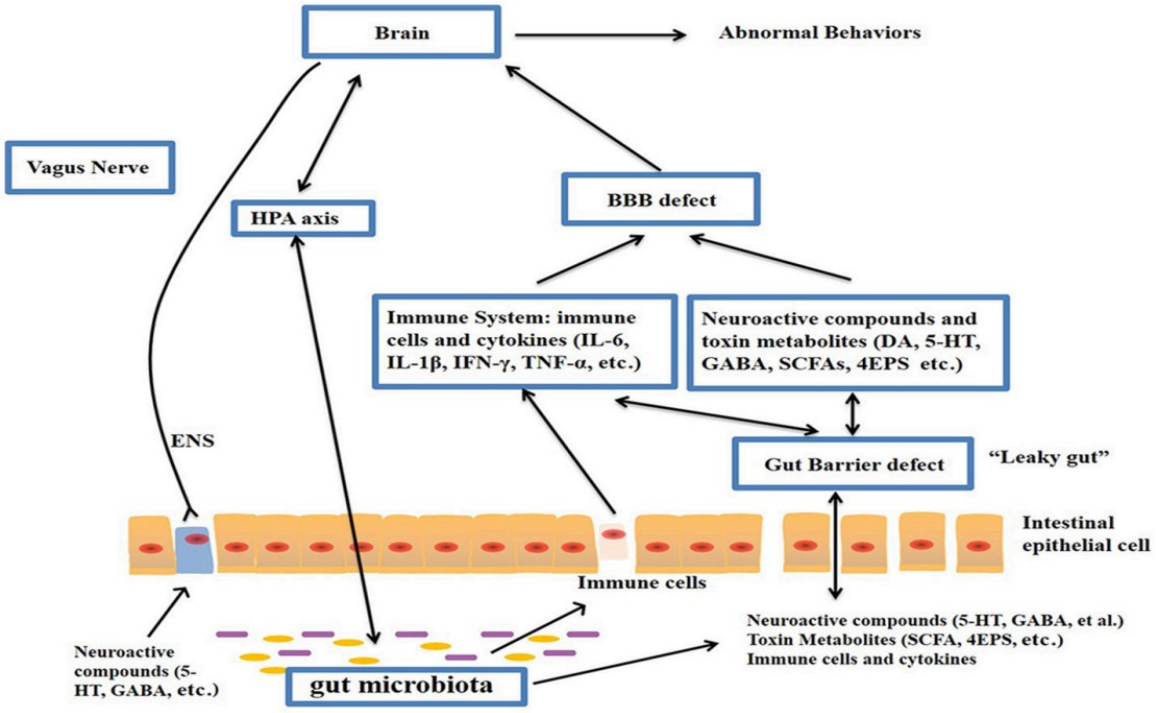

FIGURE 2.12: Communication pathways between gut microbiota and ASD. 4-EPS, 4-ethylphenyl sulfate; 5-HT, serotonin; HPA, hypothalamic pituitary adrenal; SCFAs, short-chain fatty acids; BBB, blood-brain barrier; 5-HT, 5-hydroxytryptamine; ENS, enteric nervous system; GABA, λ-aminobutyric acid; DA, dopamine. Adapted from [41].

Neuroendocrine Pathway

Neuroendocrine pathway encompasses Hypothalamic-pituitary-adrenal (HPA) axis and Neurotransmitters and neural regulator. The HPA axis works when a threat of stress exists for the body. Vasopressin and corticotropinreleasing hormone (CRH) are released by the hypothalamus under the stress conditions, and the pituitary gland releases adrenocorticotropic hormone (ACTH) under the signal of CRH and vasopressin. As a result of this, hormones originating via adrenal glands like cortisol are released under the stimulation of ACTH. Cortisol is a glucocorticoid and it exerts crucial effects on many vital organs of human body such as brain, where it regulates

the functioning of intestinal cells including epithelial cells, enterochromaffin cells, immune cells, enteric neurons and smooth muscle cells. This results in the maintenance and regulation of gut mucus, motility , permeability, and immunity.

The same intestinal cells are under the control of gut microbiota, and dysbiosis results in release of pro-inflammatory cytokines due to immune activation. These cytokines in turn may get access to the hypothalamus due to leady gut and acts on it as a stress signal [66]. Altered mRNA levels of CRH receptor and glucocorticoid receptor 1 are observed in ASD individuals implying the impairment of this pathway [67].

Neuroactive Compound Pathway

Certain important neurotransmitters like glutamate, dopamine, gamma amino butyric acid (GABA), and serotonin are regulated by gut microbes and their altered levels have been reported in ASD individuals. The excitation (glutamate) and inhibition (GABA) imbalance has also been proposed in the etiology of ASD [1].

Serotonin, a major neurotransmitter in the gut and CNS is reported to be in higher level in ASD subjects due to increased number of *Enterococcus, Escherichia, Candida, Clostridiales,* and *Streptococcus* that results in hyperserotoninemia and intestinal dysmotility.

As tryptophan is the precursor for serotonin, higher levels of serotonin consume the precursor tryptophan making it less available in the brain for serotonin production there. The serotonin in the brain regulates mood and cognition, and lesser serotonin leads to mood swings and cognitive impairment in ASD [68].

Immune System Pathway

Another pathway through which the gut can influence the brain involves the immune system. Various immune response impairments have been observed in ASD patients including the increased density of reactive microglia in prefrontal cortex of ASD brains, and enhanced microglial activity in white matter, cortical regions, and cerebellum. Maternal Immune Activation (MIA) models also confirm such neuroimmune abnormalities [69].

TABLE 2.3: Table showing pathways of communication between gut microbes and brain, and their possible roles in ASD.

Sr. No.	Name of Pathway	Possible Role in ASD	Ref.
1	Neuroendocrine pathway	Mood swings, cognitive impairments, excitation/inhibition imbalance	[37, 39]
2	Neuroanatomical pathway	Impaired GABAergic functioning	[35]
3	Toxins production	Degenerated synapses	[46]
4	Immunological pathway	Inflammation, neuroimmune abnormalities, neurotoxicity	[40, 45]
5	Metabolites Pathway	Neuronal damage, neurotoxicity, inflammation	[49–51]

Certain toxins (like LPS) produced by the pathogenic microbes, infection, stress, and some metabolites like propionic acid result in elevated intestinal permeability and altered intestinal barrier. Thus, the bacterial products are translocated across the intestinal wall and activate the immune system. The inflammatory cytokines released due to activated immune system activate the vagus system, that ends up in regulation of various activities of CNS [70]. Abnormalities in pro-inflammatory cytokines like TNF-α and IL-6 have been reported to affect the development of ASD. IL-6, a pro-inflammatory cytokine is produced by astrocytes, microglia, and neurons, and is critical for neuronal survival [71]. The, extensive elevated levels, however, are reported to cause severe pathologies in the brain including adhesion and migration of neuronal cells, that results in formation of excitatory synapses, and animal models confirm that these changes can lead to ASD related behaviors [72]. TNF-α is important to ensure synaptic plasticity, but the over expression badly affects the learning and memory related functions of synapses [73]. Studies confirm the higher levels of TNF-α in ASD brains, specifically in the frontal cortex as compared to NT ones [74]. These studies preclude the key role of neurotoxicity or neuroinhibition in ASD pathophysiology.

Toxins Pathways

Some microbes residing in the GI tract are capable of producing an enterotoxin or a neurotoxin with potential harmful effects on the CNS. One such neurotoxin is produced by *Clostridium* tetani, which resides in the stomach in inactive state but under

the dysbiotic environment, the growth of the microbe is favored, thus the tetanus neurotoxin produced is carried by vagus nerve to brain through the intestinal barrier. Once in the CNS, the neurotoxin cleaves the synaptobrevin, a membrane associated protein that ensures the synaptic vesicle stability. The synapses with cleaved synaptobrevin are unable to retain their stability and degenerate. This degeneration of the synapses is correlated with altered social behavior found in autism. [8, 48].

Metabolites Pathways

There are absolute indications for the involvement of certain gut metabolites in the pathophysiology of ASD. Various products such as phenolic compounds, Free Amino Acids (FAAs), and Short Chain Fatty Acids (SCFAs) are designated as gut metabolites. Indole produced by various microbes is absorbed in to gut, afterwards is converted in to indoxyl sulphate after undergoing oxidation and then sulphonation happening in liver. The product, indoxyl sulphate possesses the tendency to block the efflux transporters in BBB, thus certain neurotransmitters are piled up in brain [74]. Due to the dysbiotic environment in the gut, there is an overproduction of metabolites such as 3-hydroxypropionic acid, 3-hydroxyhippuric acid, and 3-hydroxy phenyl acetic acid. Such metabolites lead to autistic symptoms by curtailing the level of catecholamines in the brain [75].

Other than these, certain phenolic compounds like phenol, 4-cresol, and p-cresol are found in abundance in autistic subjects. P-cresol possess the ability to inhibit dopamine-b-hydroxylase, thus level of dopamine raises as it cannot be converted in to epinephrine owing to the inhibition of dopamine-b-hydroxylase. The increase in the level of dopamine is correlated to the abnormal behavior and neuronal damage [76].

The gut bacteria also produce SCFAs such as propionate, butyrate, and acetate, as a result of fermentation. These SCFAs can cross the BBB and bind to their receptors in the brain, and modulate neurotransmitters production including serotonin and dopamine, thus influencing the brain function. Propionate is found in higher levels in ASD subjects and it actually protects against hypertension by exerting inhibitory effects on immune cells, but the higher levels cause behavior problems similar to ASD. It

also acts as neurotoxin and inhibits the production of first substrate in electron transport chain which is Nicotinamide adenine dinucleotide (NADH), and thus degrades the nervous system. Certain antioxidants like superoxide dismutase are found to be reduced in autistic individuals, primarily due to the presence of pro-inflammatory cytokines, which in turn are generated by propionic acid [48].

Butyrate is thought to be one of the most significant SCFA as it has anti-inflammatory properties and is neuroprotective. Due to histone deacetylase inhibitor activity, it is implicated in epigenetic regulation thus promoting memory formation, and neuronal plasticity. The reduced level of butyrate in ASD subjects is speculated to affect the pathophysiology of ASD due to its neuroprotective functions. Butyrate can also alter the gene expression of tyrosine hydroxylase, thus regulating the production of dopamine, epinephrine, and norepinephrine [77]. Thus, the metabolites produced by the gut microbiota are implicated in the pathophysiology of ASD.

2.11.3.2 Microbial Composition In ASD

Several studies have been conducted to analyze the microbial composition in ASD subjects and the results obtained by different studies are inconsistent. The table showing various studies conducted with the aim to analyze the gut microbial composition in ASD subjects has been shown here in table 2.4.

TABLE 2.4: Table of various studies showing gut microbial composition in ASD subjects.

Author/ Yr/Popul.	**Autistic (n)**	**Cont. (n)**	**Outcome**	**Ref**
Finegold et al. 2002 Chicago	13	8	↑*C. aminobutyricum* *C. cocleatum* *C. clostridioforme* *C. bifermentans* *C. nexile* *C. difficile*	[78]

			C. disporicum *C. glycolicum* *C. innocuum* *C. lactifermentum* *C. orbiscindens* *C. ramosum* *C. roseum* *C. scindens* *R. torques*	
Song et al. 2004 Chicago	15	8	↑ *C. bolteae* *Clostridium* clusters I and XI	[79]
Parracho et al. 2005 UK	58	22	↑ *C.histolyticum* group (clusters I and II)	[80]
Adams et al. 2011 America	58	39	↑*Lactobacillus* Bacillus *spp* ↓ *Bifidobacterium* *Enterococcus* *Klebsiella oxytoca*	[81]
Wang et al. 2011 South Australia	23	31	↑*B. fragilis* L *Bifidobacterium* *spp. A. muciniphila*	[82]
Williams et al. 2011 *Caucasian* Hispanic African- American	15	7	↑ ratio of *Firmicutes* to *Bacteroidetes* *Protobacteria* class: Betaproteobacteria order: Clostridiales/ Bacteroidales ratio Clostridiales	[83]

			family level: Lachnospiraceae and Ruminococcaceae Alcaligenaceae IncertaeSedis 5 Methylobacteriaceae Unclassified Rhizobiales genus: Faecalibacterium Lachnopsiraceae IncertaeSedis Unclassified Lachnospiracea Bryantella ↓Bacteroidetes	
Williams et al. 2012 ND	23	9	↑ genus Sutterella,	[84]
Gondalia et al. 2012 Austrailia	51	31	no differences	[85]
Wang et al. 2013 Austrailia	23	10	↑ Sutterella, R. torques	[86]
Kang et al. 2013 ND	20	20	↓ genus: Prevotella, Coprococcus, unclassified Veillonellaceae	[87]
Angelis et al. 2013 Italy	20	44	↑ Bacteroidetes Porphyromonadaceae Prevotellaceae	[88]

Enterobacteriaceae
genus: Proteus
Shigella
Caloramator and Sarcina
specie: *Barnesiell-*
aintestinihominis
Odoribactersplanchnicus
Parabacteroides sp
Roseburia sp.
Roseburiainulinivorans
Doreasp
Turicibacter sanguinis
Odoribactersplanchnicus
and *Parabacteroides* sp
Prevotellacopri and
Prevotellaoris
Alistipes species
Parasutterella-
excrementihominis
Akkermansiamuciniphila
Bacteroides
Porphyromonas
and *Prevotella*
Pseudomonas
Aeromonas and
Enterobacteria
↓ *Firmicutes*
Fusobacteria
Verrucomicrobia
genus: Oscillospira
Sporobacter

			Subdoligranulum Enterococcus species Collinsella Fusobacterium Lactobacillus Streptococcus Lactococcus Staphylococcus Bifidobacteria Prevotella and Enterobacteria	
Tomova et al. 2014 Slovakia	10	19	↑ Lactobacillus spp Desulfovibriospp ↓ Bacteroidetes/ Firmicutes ratio	[89]
Inoue et al. 2016 Japan	6	19	↑ genus: *Faecalibacterium* ↓ genus: Blautia	[90]
Son et al. 2015 White/ Non-Hispanic Hispanic Black	59	6	no differences	[91]
Iovene et al. 2017 Italy	47	19	↑ Peptococcus Bacterioides Fusobacterium and Bacillus ↓ Lactobacillus spp less rich in number of *Clostridium* spp	[92]

Finegold et al. 2017 ND	33	21	↑ *Clostridium perfringens*,	[93]
Strati et al. 2017 Austrailia	40	21	↑ taxa: Escherichia/ Shigella and *Clostridium* cluster XVII Family: Firmicutes/ Bacteroidetes ratio genus: Corynebacterium Collinsella Lactobacillus Dorea ↓ Bacteroidetes genus: Veillonella Dialister Parabacteroides Alistipes Bilophila	[94]
Kushak et al. 2017 ND	21	13	↑genus Burkholderia Actinomyces Peptostreptococcus Ralstonia ↓ genus Neisseria Devosia Prevotella Bacteroides Streptococcus species: *Bacteroides Bvulgatus* *Escherichia coli*	[95]
Lee et al. 2017	20	28	↑ phylum: Firmicutes Verrucomicrobia	[96]

Korea	family: Streptococcaceae
	Verrucomicrobiacea
	Clostridiaceae
	an unclassified
	Clostridiales
	and Eubacteriaceae
	genus:Streptococcus
	Akkermansia
	Jeotgalicoccus
	Desulfovibrio
	Oscillospira
	Rhodococcus
	alomonas
	an unclassified
	member of family
	Comamonadaceae
	an unclassified
	member of f-S24-7
	↓ phylum: Proteobacteria
	Cyanobacteria
	Armatimonadetes
	order: Streptophyta
	family: Alcaligenaceae
	Sphingomonadaceae
	Rhizobiaceae
	Verrucomicrobiaceae
	genus Pseudomonas
	Sphingomonas
	Agrobacterium
	Achromobacter
	Roseateles

Luna et al. 2017 Columbus, Ohio, US	ASD	14	40	↑ Clostridiales *Clostridium* lituseburense Lachnoclostridiumbolteae Lachno*Clostridium* hathewayi *Clostridium aldenense* *Flavonifractorplautii* *Terrisporobacter* species ↓ Doreaformicigenerans Blautialuti Sutterella	[97]
Kang et al. 2018 America	23		33	↓ prevotellacopri Feacalibacteriumprausnitzii Haemopliusparainfluenzae	[98]
Zhang et al. 2018 China	35		41	↑ Phylum Bacteroidetes/ Firmicutes ratio ↓ genus:Veillonella Eschericia and Streptococcus	[99]
Coretti et al. 2018 Italy	11		14	↑ Phylum: Bacteroidetes and Proteobacteria Bacteroidetes/ Firmicutes ratio *Faecalibacterium*prausnitzii genus: B. vulgatus B. uniformis	

			P. distasonis Enterobacteriaceae and Pasteurellaceae Ruminococcaceae Faecalibacter- iumprausnitzii Oscillospira ↓ phylum: Actinobacteria family: Coriobacteriaceae Actinomycetaceae Bifidobacteriaceae Streptococcaceae Gemellaceae genus: Coriobacteriaceae Actinomyces Corynebacterium Eggerthellalenta *Bifidobacterium longum* Streptococcus	
Rose et al. 2018 United States	50	6	↑ famiy: Bacteriodaceae Lachnospiraceae Prevotellaceae and Ruminococcaceae	[100]
Ma et al. 2019 China	45	20	↑ specie: *Clostridium* clostridioforme ↓ family: Acidaminococcaceae Genus: Lachno*Clostridium* Tyzzerella subgroup 4 Flavonifractor	[101]

			unidentified Lachnospiraceae	
Liu et al. 2019 China	30	45	↑ Phylum: Acidobacteria Taxa: Veillonellaceae Enterobacteriaceae Fusobacterium Barnesiella Coprobacter Actinomycetaceae Genus: Megamonas ↓ Phylum Firmicutes Taxa: Ruminococcaceae Streptococcaceae Enterococcus Hungatella Aggregatebacter Holdemania Anaerotruncus [Eubacterium] fissicatena group Phocea Anaerostignum ↓Genus *Faecalibacterium* Lachnospiraceae Corynebacterium Blautia Faecalitalea Campylobacter Agathobacter Bacillus Alistipes	[102]

			Ezakiella Murdochiella Finegoldia Anaerococcus Lawsonella Candidatus saccharimonas Peptoniphilus Alisonella	
Wang et al. 2019 China	43	57	↑ phylum: Actinobacteria Eggerthellalenta and *Clostridium botulinum* *Clostridium botulinum* Ba4 strain 657 *Clostridium botulinum* A3 strain Loch Maree *Clostridium cellulolyticum* *Eggerthellalenta* and Eggerthellalenta DSM 2243 *Klebsiella pneumoniae* *subsp. pneumoniae* *Klebsiella pneumoniae* ↓ *Betaproteobacteria* *Bacteroides vulgatus* *Campylobacter* *jejuni* subsp. *jejuni* ICDCCJ07001 *Campylobacter jejuni* subsp. jejuni 81-176	[103]
Plaza-Diaz et al. 2019	48	19	↑ phylum: Actinobacteria and Proteobacteria	[104]

ND			Family: Corynebacteriaceae and Clostridiales family XVII Microbacteriaceae Thermoanaerobacteraceae Bifidobacteriaceae Thermoactinomycetaceae Desulfohalobiaceae Bacillaceae Enterobacteriaceae Enterococcaceae *Clostridium* difficile ↓ family: Lachnospiraceae	
Kong et al. 2019 Israel, Boston	20	31	↑ class:Bacilli Firmicutes/ Bacteroidetes ratio Proteobacteria	[105]
Sun et al. 2019 China	9	6	↑order Ruminococcaceae ↓Class Bacteroidales Selenomonadales order Prevotellaceae	[106]
Zurita et al. 2019 Ecuador	25	35	↑genus Bacteroides Akkermansia Coprococcus ↓genus Lactobacillus Ruminococcus	[107]
Hazan et al. 2020 United States	1	3	↑ Phylum Firmicutes/ Bacteroidetes ratio Proteobacteria ↓Actinobacteria	[108]

Hua et al. 2020 China	120	60	↓ Genus: *Faecalibacterium* r and Agathobacte	[109]
Dan et al. 2020 China	143	143	↑ Phylum Firmicutes/ Bacteroidetes ratio Firmicutes Genus Proteobacteria Actinobacteria Dialister Escherichia-Shigella Bifidobacterium ↓Genus Bacteroidetes Prevotella 9 Megamonas Ruminococcus 2	[110]
Ding et al. 2021 Austrailia	77	50	↑Class Coriobacteriia Order Enterobacteriales Coriobacteriales family (Enterobacteriaceae) unidentified Clostridiales Coriobacteriaceae unidentified Lachnospiraceae unidentified Clostridiales unidentified Erysipelotrichaceae genus Dorea Collinsella Lachno*Clostridium*	[111]

			↓Order Gammaproteobacteria family Bacteroidaceae Burkholderiaceae genus Bacteroides *Faecalibacterium* Parasutterella Paraprevotella	
Shih-Chen Fu et al. 2021 China	40	40	↑Phylum Firmicutes family Lactobacillaceae Peptostreptococcaceae ↓Phylum Bacteroidetes/ Firmicutes Bacteroidetes	[112]
Ye et al. 2021 China	71	18	↑genus Eisenbergiella Klebsiella Megasphaera *Faecalibacterium* Blautia ↓genus Akkermansia Escherichia Veillonella Dialister Bifidobacterium Citrobacter Streptococcus Rumini Clostridium_6 Ruminococcaceae UCG_002 Eubacterium_ Shigella	[113]

			Rumini*Clostridium*_5 Provindencia coprostanol	
Ding et al. 2021 China	25	20	↑Phylum Firmicutes Genus *Faecalibacterium* Prevotella Subdoligranulum Ruminococcus ↓Phylum Actinobacteria Genus *Bifidobacterium*	[114]
Lou et al. 2021 China	773	429	↑Genus Acidaminococcus Veillonella Clostridioides Eubacterium Paraprevotella Megasphaera Coprobacter Parasutterella [Eubacterium] xylanophiulm group Moryella	[115]
Zhang et al. 2021 China	21	21	↑ Phylum Firmicutes/ Bacteroidetes ratio families: L achnospiraceae and Ruminococcaceae Genera: Lachnospiracea incertae sedis Ruminococcus Blautia	[116]

			and Holdemanella Prevotella copri	
Zilin et al. 2021 China	138	60	↑Genus Bacteroides *Faecalibacterium* Sutterella Collinsella ↓Genus Prevotella Coprococcus Desulfovibrio	[117]
Cao et al. 2021 Korea	45	41	↑ genus *Clostridium* Desulfovibrio Streptococcus Neisseria Bacillus Streptomyces ↓family Lachnospiraceae. Genus Bacteroides	[118]
Ha et al. 2021 Korea	54	38	↑ Bacteroidetes to Firmicutes ratio Phylum Actinobacteria class Actinobacteria order Bifidobacterioles family Bifidobacteriaceae genus *Bifidobacterium.* ↓ phylum Bacteroidetes class Bacteroidia order Bacteroidales family Bacteroidaceae genus Bacteroides	[119]
Jendraszak et al. 2022	33	16	↑Specie *Bifidobacterium* ↓ Specie Klebsiella spp.	[120]

China				
Hong et al.	36	25	↑ Specie Eubacterium hallii	[121]
2022			Anaerostipes caccae	
China			*Bifidobacterium bifidum*	
			↓ Specie *Haemophilus parainfluenzae*	
			Roseburia intestinalis	
			Rothia mucilaginosa	
			Akkermansia muciniphila	
Wong et al.	92	112	↑Phylum Firmicutes: Bacteroidetes	[122]
2022			genus *Bifidobacterium*	
Taiwan			Dorea	
			Blautia	
			↓genus Bacteroides	
			Clostridium	
			sensu stricto 1	
			Parabacteroides	
			uncultured	
			Eggerthellaceae	
			Collinsella	
			Alistipes	
			Sutterella	
Chen, Y. C et al.	82	31	↑Genus Ruminococcus torques group	[123]
			Fusobacterium	
2022			specie Bacteroides plebeius	
Australia			DSM 17135 ↓genus	

Ruminococcus
torques group
Ruminococcaceae
UCG 013
Parasutterella
Clostridium
sensu stricto 1
Turicibacter
Intestinimonas
butyriciproducens
Ervsipelotrichaceae
UCG 003
specie *Clostridium*
spiroforme DSM 1552

ASD is a multifactorial disorder and both genetics and environmental factors contribute in the onset. As genetics is only involved in 10-20% of the cases, environment seems to play a promising factor in the pathophysiology. Among the environmental factors, gut microbiota is given the priority as the most commonly observed co morbidity in ASD children is GIS. The time frame of gut microbiota establishment and stabilization also coincides with that of nervous system development.

Gut microbiota-brain axis is a direct cross-talk between the gut and CNS, emphasizing the direct connections between the two vital body systems and implicating that both the gut and the brain directly influence the physiology of each other. Various studies have shown the variations in gut microbial composition in children with ASD when compared to their siblings, or other NT developing children. The results obtained by different research groups to assess the gut microbial composition of ASD subjects as opposed to their siblings or healthy children are inconsistent and no conclusive findings can be drawn but owing to the changes and variations in gut microbial composition,

and impact of gut microbes in human health, it is provoking to explore this area more extensively to better underpin the etiology of ASD.As no elucidation of the pathways and mechanisms by which these microbes add to ASD exists, we need to quest for the gut microbial metabolites and their role in association with the onset or the underlying pathophysiology of the disorder. These gut microbial metabolites could further be used for early diagnosis so that the financial, and emotional disease burden could be reduced.

Chapter 3

Research Methodology

The present study has been aimed to explore the pathophysiology of ASD by exploring the interaction of gut microbiota with ASD. The first objective to achieve the aim has been to analyze the variations in gut microbial composition. To address the issue of in-consistent results regarding the gut microbial composition in ASD children as opposed to their age-matched neurotypical controls, and in an attempt to include more recent studies on the issue, a meta-analysis has been conducted so that the statistically significant conclusions regarding variations in gut microbial composition could be drawn. The results obtained are speculated to be helpful in devising new therapeutic strategies for ASD individuals.

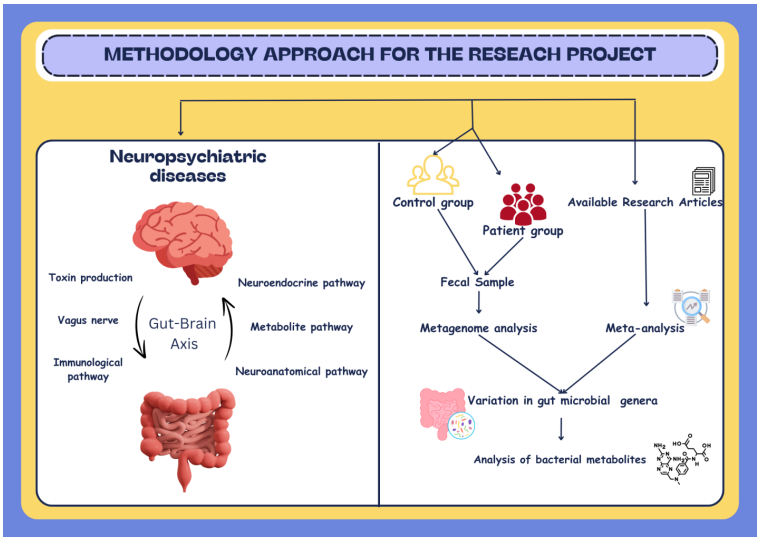

FIGURE 3.1: The methodology used in the current research project.

3.1 Meta-analysis

Meta-analysis refers to the statistical analysis of the various results, obtained from different individual studies in order to have integrated outcomes. Meta-analysis is conducted to systematically analyze the data from already executed research studies to statistically conclude about included research studies. In order to check the strength of evidence on a disease or some treatment, meta-analysis is the go to strategy of the researchers. This procedure is adopted to assess whether an effect exists, or the effect has positive or negative aspects. The overall theme of the methodology is to concise over a single summary estimate of the effect. The conclusions drawn by the meta-analysis possess some key benefits like answering those questions that are even not evident from individual studies, resolving the conflictive conclusions obtained from individual studies, and boost up the accuracy of summary estimate of the effects [124].

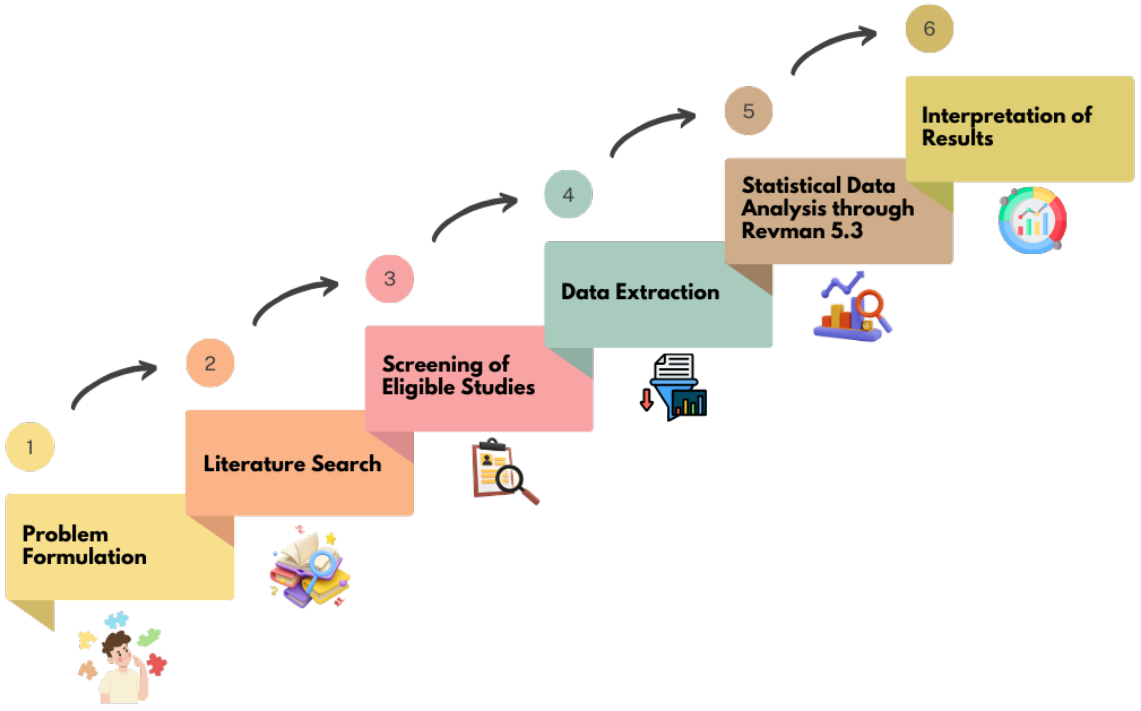

FIGURE 3.2: The methodology used for Meta-analysis.

3.1.1 Standardized Mean Differences

Standardized mean difference (SMD) estimation is primarily used for continuous data. SMD is used when different researches under study measure the same outcome but different ways and methods are used in different studies to measure that same outcome.

Under such conditions, the primary need is to make the available data standardized to a common scale before these studies are combined together in order to derive statistically significant results [125].

3.1.2 Assigning Weights to the Studies

The methods used in meta-analysis include weighting of the studies. The value of evidence of any particular study is basically reflected by the weighting. It is a general practice that smaller the study, lesser is the weight to the overall estimate of the effect, because the studies are generally weighted according to the inverse of their variance. When the sample sizes are larger, more weight is given to the studies [126].

3.1.3 Statistical Models Used in Meta-Analysis

One of the critical decisions to be made while conducting meta-analysis is which statistical model is to be used, whether the random effect or the fixed effect model was applied.

Fixed-Effects Model: The fixed-effects model is based on the assumptions that all the studies included in the meta-analysis bear true effect size. Thus, the sole source of variations obtained are the sampling error, and arise within the study [127].

Random-Effects Model: On the other hand, the random effect model lies on the stance that the study differences (heterogeneity) result in the variations in the true effect from one study to another. Generally, while considering the uncertainty resulting

from heterogeneity among the studies, the random-effects model is the most practical and suitable model to be employed [128].

3.1.3.1 Heterogeneity

Heterogeneity is an interpretation of the differences that exists between studies and reflected in study outcomes. In order to measure the inconsistency of studies' results, heterogeneity is calculated.

Heterogeneity is better quantified by inconsistency index I^2, which shows the percentage of variations across the studies. The values of I^2 ranges between zero to 100%. and a value of >75% denotes substantial heterogeneity among the studies [129]. This meta-analysis has been conducted keeping in view the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [130].

3.2 Literature Search Design

The databases quested to conduct the meta-analysis were Scopus, Web of Science, PubMed, Cochrane databases, and Science database. Those studies were searched that identified the variations in gut microbial profile in children with and without ASD up till 31st July 2023 using search terms:

(ASD *OR autism * OR autism spectrum disorder * OR autistic disorder*) AND (gut *OR gastrointestinal* OR dysbiosis* OR intestine*) AND (microbiota *OR microflora* OR stool* OR fecal* OR microbiome*).

3.2.1 Inclusion Standards for Studies

The searched articles were assessed for their titles and then the abstracts, and full text of relevant studies were minutely examined and thoroughly read. Inclusion and exclusion criteria is shown in figure 3.3.

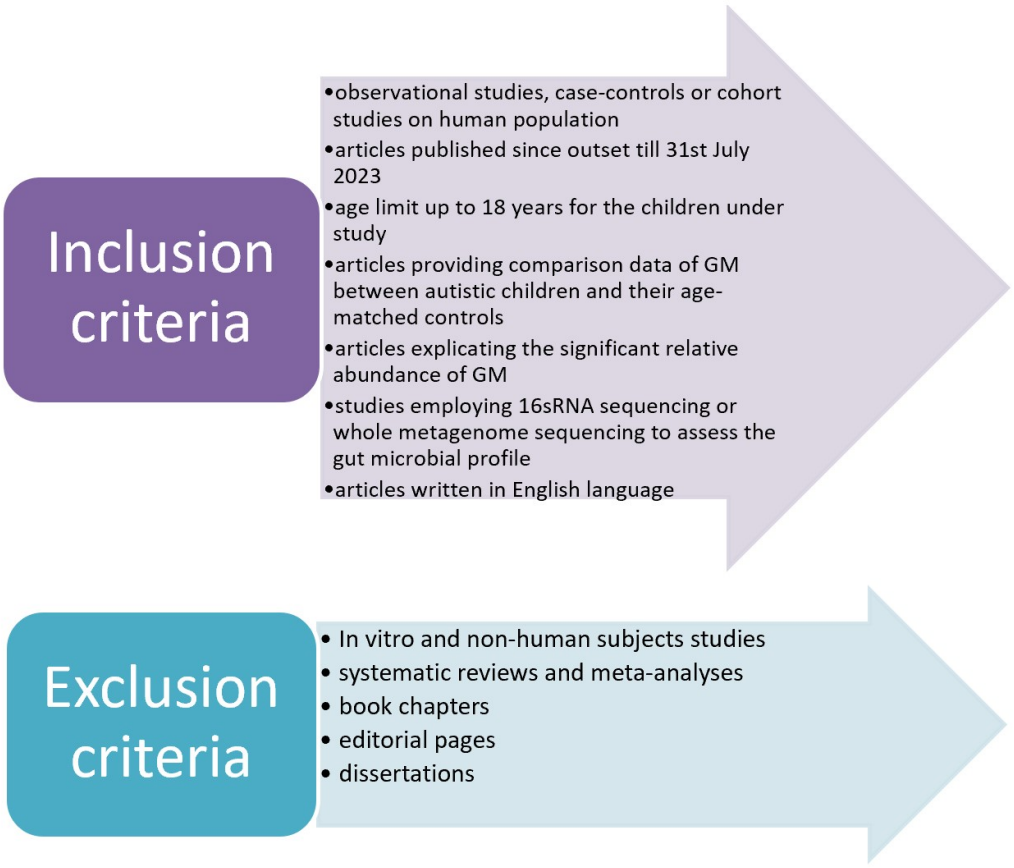

FIGURE 3.3: Inclusion and exclusion criteria for studies of meta-analysis.

3.3 Data Extraction

The included studies were carefully assessed to draw data such as the first authors' surname along with the publication year, population under study, study design, details of ASD subjects and control (sample size, gender, age), DNA extraction sample, details of microbiota (microbiological assessment, outcome in terms of phylum and genus level differences, unit), diagnosis of ASD and diagnostic tool used for the assessment, and co-morbid conditions.

Additionally, variations in sequencing techniques, bioinformatics pipelines, and statistical approaches were noted to ensure methodological consistency. Studies were also examined for potential biases, limitations, and the robustness of their findings. This comprehensive evaluation facilitated a thorough comparison of results across different studies, contributing to the reliability of the overall analysis.

3.3.1 Quality Assessment of Included Studies

All included studies were systematically reviewed to ensure data accuracy and reliability. A rigorous double-checking process was employed to validate the extracted information. Comprehensive analysis was conducted to assess methodological consistency and validity. This approach ensured the integrity and reproducibility of the findings.

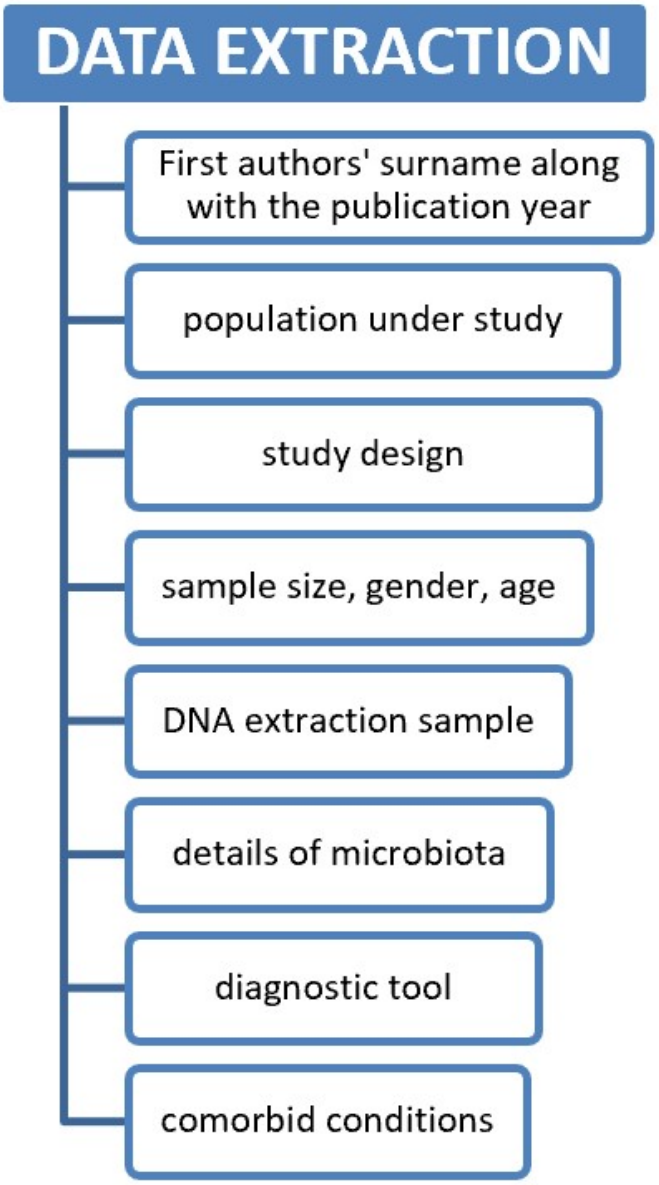

FIGURE 3.4: Data extraction variables used in meta-analysis.

3.4 Statistical Data Analysis

Relative abundance (RA) of various bacterial phyla and genera in ASD children and neurotypical controls was mentioned in the included studies. RA refers to the percent composition of an organism or a substance of a specific type as compared to the overall organisms or substances present in a specific habitat, ecosystem, or biome [131].

Mean, Standard Error (SE) and Confidence Interval (CI) was also mentioned for these measurements in some of the studies. Mean denotes the mathematical average of a set of two or more numbers. SE refers to a mathematical tool that is used to calculate variability. SE is equal to the ratio of the standard deviation to the square root of the sample size.

$SE = SD/\sqrt{N}$ Where the standard deviation is denoted by SD, and the sample size is expressed by N [132].

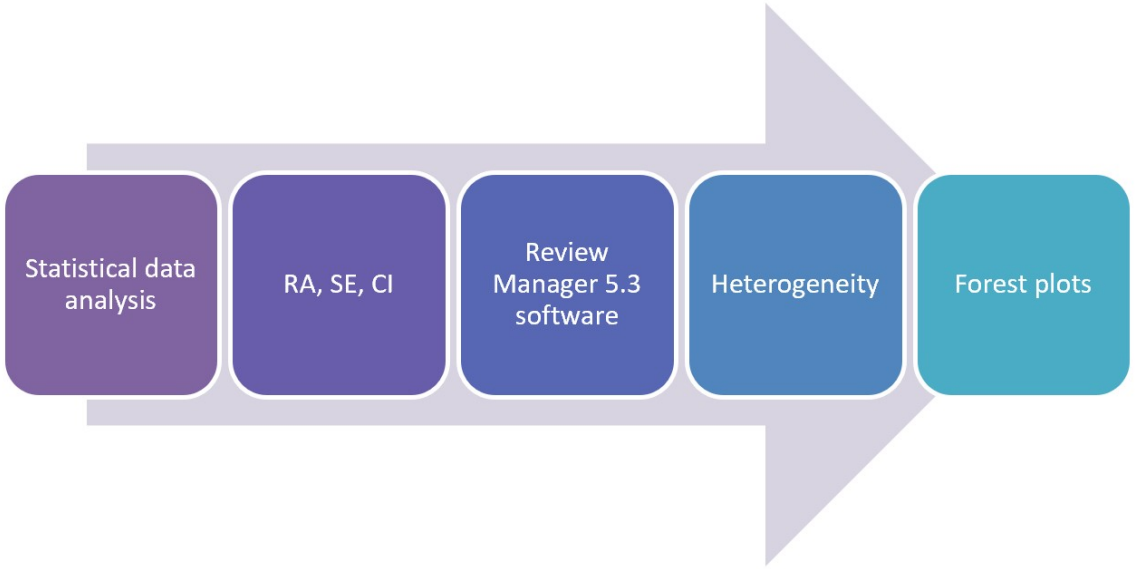

FIGURE 3.5: The sequence of steps performed in meta-analysis where RA stands for Relative abundance, SE stands for Standard error, and CI stands for Confidence interval.

CI is a means to tell about the probability. It is used to calculate the interval, is taken by the observed data, and the unknown parameter is depicted by it in terms of its actual value [133]. RA and SE is used to standardize the data in order to evaluate the

differences in bacterial phyla and genera in children with ASD and healthy controls. Variation in the percentages of gut bacteria between children with ASD and healthy controls was identified against each bacterial phylum and genera so that the RA of gut bacteria in ASD children and neurotypically developing children could be known.

Review Manager 5.3 software was used by choosing the inverse-variance method to weight each effect size so that random-effect meta-analysis could be conducted. Statistical heterogeneity among the effect sizes was analyzed by computing I^2 index. Values of observed I^2 equal to 25%, 50%, and 75%, related to lower, moderate, and higher heterogeneity, thus larger the value of I^2, larger is the heterogeneity [134]. Forest plots were constructed to visualize the results against each analyzed bacteria by computing weighted mean effect size with a 95% CI. The analyses in which the p value was <0.05 were considered as statistically significant.

Forest plot is used to display the results from various studies that address the same question in a graphical way [135]. A box in the forest plot represents each included study, and a horizontal line specifying 95% CI passes through it. The size of the box represents the weight of the study. The larger box shows that the included study provides more information, and hence given more weight.

The overall pooled effect of the studies is represented by a diamond. The forest plot also contains a vertical line, which is basically the line of no effect. The general rule says that there will be no significant statistical difference if the horizontal line of 95% CI passes through the line of no effect. On the other hand, if the line of 95% CI does not cross the line of no effect, the significant statistical difference exists. Heterogeneity among the studies is calculated through I^2 statistics. If the values range from zero to 40%, it shows that the heterogeneity might not exist. The moderate heterogeneity is depicted if the values range from 30%-60%. The substantial heterogeneity is represented if the values range from 50%-90%, where as 75%-100% show off considerable heterogeneity.

The next objective of the present study has been to assess the variations in gut microbial diversity in autistic children as compared to the healthy controls. Various

studies have shown the variations with respect to gut microbial composition in ASD as opposed to healthy populations, and numerous species have been shown to cause microbial dysbiosis in ASD subjects. However, inconsistent results have been drawn by different studies regarding the gut microbial composition.

Recently available systematic review and meta analysis concluded a lower percentage of *Bifidobacterium* and Coprococcus and a higher relative abundance of the genera *Faecalibacterium*, *Clostridium*, *Parabacteroides*, *Bacteroides*, and *Phascolarctobacterium* in ASD affected children as compared to healthy controls [8]. Therefore studies focusing on gut microbial composition could help researchers to provide greater insight in to the etiology of multifactorial nature of ASD.

This study was conducted to assess the fecal metagenomic profile of healthy and autistic children in order to ascertain the composition of microbiota in both groups. As far as we are aware, none of the studies have been conducted in Pakistani population covering this issue.

16S rRNA sequencing of fecal samples of 2 ASD children with ages between 2 to 9 years and 2 age-matched neurotypical children was performed in an endeavor to explicitly delineate the differences in gut microbes of ASD children and healthy controls. The specified bacteria in this study could help in the treatment opportunities and possible relief from the GI symptoms in children with ASD in Pakistani population.

3.5 Metagenomics

Metagenomics refers to the molecular tool to study the communities of micro- organisms by analyzing their DNA without the need for any pure culture. The phylogenetic associations of the sequenced gene with the already known taxonomic entities of microorganisms in the databases can be quested through this methodology. This approach allows researchers to explore microbial diversity in various environments, including soil, water, and the human gut. It provides insights into microbial functions, interactions, and potential roles in health and disease. Additionally, metagenomics

has revolutionized the discovery of novel genes, enzymes, and bioactive compounds with biotechnological and medical applications [136].

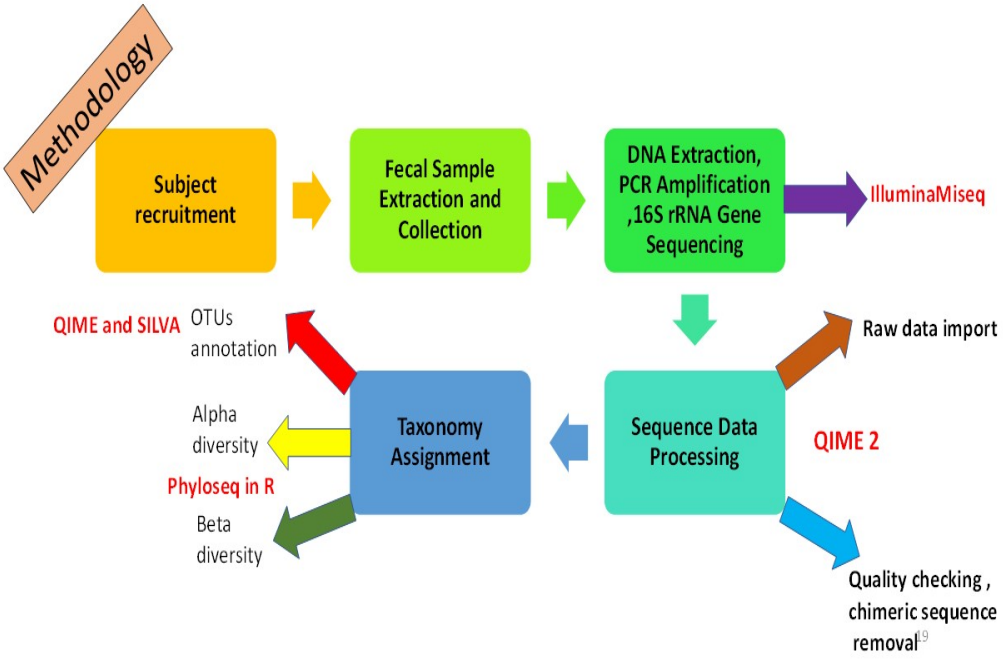

FIGURE 3.6: Methodological steps used in metagenome analysis and computation of alpha and beta diversity.

3.6 Phylogenetic Clusters

Certain phylogenetic clusters like 16S rRNA gene sequence are addressed to carry out the procedure, and the estimates of species abundance of various microbes are assessed by comparing the operational taxonomic units (OTUs) in the particular environment or habitat [137].

3.7 Operational Taxonomic Units

Operational Taxonomic Units (OTUs) refer to classifying the groups of closely related organisms. Organisms can be classified based on the sequence similarities. Usually the similarity threshold of 97% is adjusted for OTUs for better classification of organisms.

While considering, 16 S rRNA metagenomics, OTUs are considered as the closely related sequences of 16 S rRNA marker gene sequence. A taxonomic unit of genus or specie is represented by each cluster, which primarily depends on the threshold of sequence similarity. Generally, to classify the bacteria at genus level, sequence similarity threshold of 97% is taken to define OTU clusters [138].

3.7.1 OTU Table

A OTU table contains rows and columns. The rows in the table detail the genus or specie specific taxonomic unit. The columns on the other hand represent the samples. Thus, the sequence numbers that are found in each sample with respect to each taxonomic unit are represented in the OTU table [137].

The most conserved bacterial taxonomic marker is the 16S rRNA sequence, thus it is extensively used for phylogenetic analysis. In order to spot out the sample's taxonomic profile of microbial communities, the 16S rRNA sequence is frequently employed. This sequencing approach helps identify and classify bacterial species within complex microbial ecosystems.

3.8 16S rRNA

The size of 16S rRNA is 1500 base pairs (bp), and it has nine highly conserved and nine highly variable regions (V1–V9). The conserved regions serve as universal markers for bacterial identification, while the variable regions provide species-specific signatures, enabling taxonomic classification.

3.8.1 Conserved Entities in16S rRNA Sequence

The conserved entities in the 16S rRNA sequence bind to the primer during PCR amplification, ensuring the accurate and efficient amplification of bacterial DNA.

3.8.2 Hypervariable Regions of 16S rRNA Sequence

On the other hand, the sequence diversity in microbes is recognized through the hypervariable regions [139].

3.9 Illumina Sequencing

The taxonomic classification can be established by platforms like Illumina sequencing. These platforms employ V3 and V4 regions of 16S rRNA sequence and compare them with already existing, and publically available databases like SILVA, Ribosomal Database Project (RDP), Greengenes or NCBI. SILVA is the comprehensive online database that contains aligned RNA sequences from Bacteria, Archaea and Eukaryota. This database is considered an ideal reference for taxonomic classification [140].

3.10 Statement Pertaining to Ethics

The ethical review committee at Capital University of Science and Technology, Islamabad issued approval for the present research study. The study participants showed their acceptance for the project and written informed consent was taken.

3.11 Study Participants Recruitment

Participants' metadata including prenatal and postnatal factors, background history, age, gender, eating habits, growth patterns, and GI symptoms was obtained through questionnaires. The recruited subjects were previously diagnosed with ASD through an expert neurologist and psychiatrist through DSM V criteria. The inclusion criteria was non-syndromic autism, no underlying disease except ASD diagnosed, not any previous trauma or accident or any other disease like meningitis that may play a role

in brain dysfunction.

All participants were not taking any antibiotics, prebiotics, probiotics, and anti-inflammatory drugs for the past three months. Healthy children were neurotypically growing and lacking any ASD core symptoms. Table 3.1 shows the study incumbents with respect to various characteristics.

TABLE 3.1: Summary of study characteristics.

Characteristics	**ASD (1)**	**ASD (2)**	**NT (1)**	**NT (2)**
Gender	Male	Male	Female	Male
Age (Year)	2.9	7.5	8.6	5.9
Method	Meta-genome	Meta-genome	Meta-genome	Meta-genome
Paternal educational level	Graduate	Post graduate	Post graduate	Post graduate
Maternal educational level	Post graduate	Graduate	Post graduate	Post graduate
Are the parents relative	No	Yes	No	No
ASD affected individuals in family	No	No	No	No
Any gestational disease/infection ?	No	Urine infection, candida infection	No	No
Medicines taken during gestation	Suppl-ements	Suppl-ements	Suppl-ements	Suppl-ements

Food intolerances not present previously?	No	No	No	No
Alcohol consumption?	No	No	No	No
Smoking?	No	No	No	No
Preterm / on term birth	On term	n term	On term	On term
Mode of delivery	C.section	Natural birth	Natural birth	Natural birth
Age of mother at the time of birth?	20-25 years	25-35 years	25-35 years	25-35 years
Weight of the child at the time of birth?	3 kg	3.3 kg	3 kg	3.1 kg
First feed?	Formula milk	Mother feed	Honey	Mother feed
Feeding pattern	Formula milk	Mix feed	Mix feed	Mix feed
Weaning Age	8 months	6 months	6 months	7 months
Difficulty accepting new taste during weaning	Yes	Yes	No	No
Any currently used special diet	Gluten free diet	NA	NA	NA
Age of diagnosis of ASD	2-3 years	1-2 years	NA	NA
Observations at the time of diagnosis?	Speech delay	Non-responsive	NA	NA

Severity level	Level 1	Level 2	NA	NA
Cry characteristics	A bit unusual	Satis-factory	Satis-factory	Satis-factory
Sleep characteristics	Satis-factory	A bit unsatis-factory	Satis-factory	Satis-factory
Physical growth trend	Satis-factory	Satis-factory	Satis-factory	Satis-factory
Cognitive growth trend	Unsatis-factory	Unsatis-factory	Satis-factory	Satis-factory
Picky Eater	Yes	Yes	No	No
Dysphagia	Yes	No	No	No
Recurrent abdominal pain	No	Yes	No	No
Constipation	No	Yes	No	No
Diarrhea	No	Yes	No	No

***ASD 1 refers to autistic child 1, ASD 2 autistic child 2, NT denotes neurotypically growing child 1, and NT 2 denotes neurotypically growing 2.**

Flowchart of the methodology for determination of fecal metagenomic profile in ASD and NT (neurotypically) growing children. OTU stands for Operational Taxonomic Units is mentioned in fig 3.7.

3.12 Fecal Sample Collection

Fecal samples of four children aged 2–9 years were collected, including two samples from ASD patients and two from healthy controls/neurotypically (NT) children.

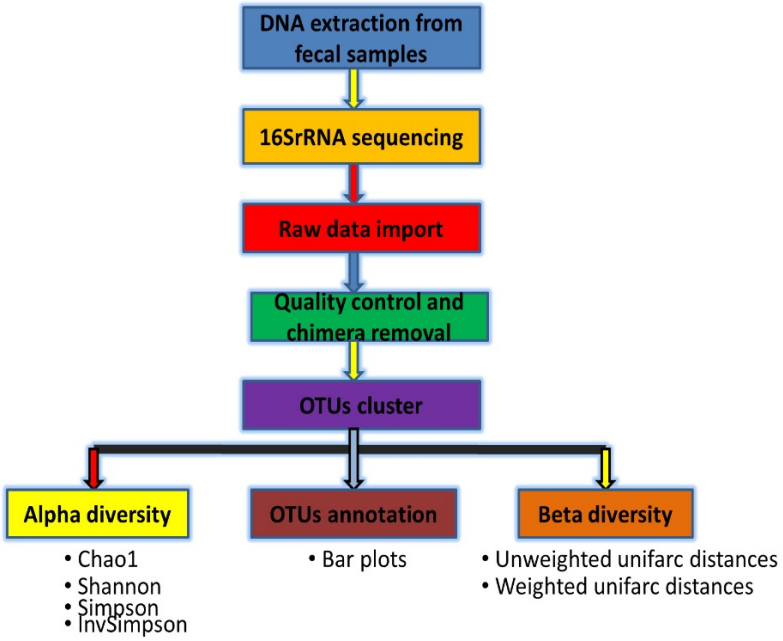

FIGURE 3.7: Flowchart of the methodology for determination of fecal metagenomic profile in ASD and NT (neurotypically) growing children. OTU stands for Operational Taxonomic Units.

The samples were taken in 500 ml Falcon tubes, each containing 6 ml of Phosphate-Buffered Saline (PBS). Ten grams of each sample was added to the respective Falcon tube. The PBS solution was prepared by dissolving one PBS saline tablet (0.96 mg) in 30 ml of distilled water, and a total of 100 ml was prepared by adding 70 ml of additional distilled water.

The samples were initially stored overnight at -20°C and then transported to the laboratory, where they were stored at -80°C before further analysis to ensure sample integrity and prevent microbial degradation.

3.13 DNA Extraction, PCR Amplification and 16S rRNA Gene Sequencing

Bacterial genomic DNA was extracted through standard procedure of phenol-chloroform method [141]. The extracted DNA was run on agarose gel and DNA quantification was measured using Thermo scientific Multi Skan Go Instrument.

3.14 Illumina MiSeq Sequencing

Initially, 200 ng genomic DNA was taken and amplification of V3–V4 regions associated with 16S rDNA gene was done. The purified PCR amplicons were sequenced on Illumina Mi-Seq platform. Paired-end method was used to construct fragment library by using paired end method in order to have paired end sequencing. The base quality scores of Illumina HiSeq (TM) /MiSeq platforms are expressed in Q Phred.

3.15 Sequence Data Processing

3.15.1 Import of Raw Data

Paired end reads (FASTQ) that were raw in characteristics, were taken from the original DNA fragments and then imported in Quantitative Insights Into Microbial Ecology (QIIME) version 2 2021.4 software. This tool has emerged to provide accurate and in time data in metagenomic analysis. The manifest file method was used in order to import paired end reads pertaining to all samples . Quality check and removal of chimeric sequence DADA2 denoising method was employed to carry out the quality filtering, denoising as well as removal of chimeric sequences. Read truncation method was in practice with the following criteria in order to obtain same read length: (i) truncation length of upto245 bp (ii) minimum abundance of 8 counts.

3.15.2 Taxonomy Assignment

After the detection of chimeric sequences for 16S rRNA, SILVA (https://www.arb silva.de/download/archive/qiime) was employed as a reference standard databases for comparison.The Naïve Bayes classifier and q2-feature classifier plugin in QIIME

2 were used to annotate taxonomic features (OTUs, genus, species, phylum) based on 97% similarity OTUs from the reference database. Alpha diversity was assessed using

the Shannon index, while beta diversity was analyzed via weighted and unweighted UniFrac with principal coordinate analysis (PCA) in phyloseq (R) [142].

3.15.3 Alpha Diversity

Alpha diversity is defined by the biodiversity in a specific area or location or habitat. It is expressed by the number of species and denotes specie richness.

3.15.4 Shannon Index

Shannon index is widely used index for measuring biodiversity between various habitats. The values of Shannon index ranges from 0 to 5, usually the range is 1.5 to 3.5. It is denoted by H. The higher the value of H, the higher the species diversity. Lowe value of H represents lower species diversity. The habitat or community is supposed to have only one species if the value of H is zero [143].

3.15.5 Beta Diversity

Beta diversity refers to the diversity of specie between different ecosystems. It denotes the number of species that are specific to one particular ecosystem as compared to another ecosystem [144].

3.15.6 Unique Fraction Metric

Unique Fraction Metric (UniFrac) is employed for the comparison of biological communities. UniFrac uses phylogenetic distances between the organisms under study and provides information on the relative relatedness of members. It helps assess microbial community similarities and differences across various samples. This metric is widely used in microbial ecology studies, including gut microbiota research, to understand shifts in microbial composition under different conditions [145].

3.15.7 Weighted UniFrac

Weighted UniFrac is the quantitative version of UniFrac which considers the abundance of the organisms being study [146].

3.15.8 Unweighted UniFrac

Unweighted UniFrac refers to the qualitative variant of UniFrac where the presence or absence of observed organisms is taken in to account [147].

3.16 Statistical Analysis

R (http://cran.r-project.org/) was used to perform the statistical analysis. Values of p that were lesser than 0.05 were considered as significant. The third objective of the present study has been to delineate the possible mechanism/pathways through which gut microbiota could be involved in the pathophysiology of ASD.

To delineate the etiology of this complex disorder, and to offer treatment opportunities, the underlying mechanisms and pathways through which gut-microbiota can potentially coordinate with brain and results in ASD behavior are necessary to be explored.

However, there is uncertainty in explaining the causes, and pathways that could show the association of gut microbiota in the onset, development, or severity in ASD symptoms and behaviors. In order to explore the involvement of gut microbiota in ASD, software based techniques have been used for the assessment of metabolites produced by the bacterial species. This study is designed with an effort to identify the metabolites produced in the gut by the various microbes that have been prioritized in the current project through meta-analysis and metagenome analysis by using various softwares. Afterwards, the pathway analysis of short-listed metabolites has been conducted through Reactome and KEGG pathway.

3.17 Identification Gut Bacterial Metabolites via Databases

The current meta-analysis statistically expressed the variations in four bacterial genera. These included *Clostridium*, *Faecalibacterium*, *Bifidobacterium*, and Coprococcus. Moreover, the results of metagenome analysis pointed to the differential expression of *Lachnospiraceae UCG-004*. The primary objective of our study on autism-related metabolites involves the identification and analysis of potential metabolites produced by bacterial species associated with autism spectrum disorder (ASD).

For this purpose, our methodology consists of

1. Data Collection and Preparation
2. Data Processing using Python
3. Metabolic Pathway Analysis

FIGURE 3.8: Autism-related Gut Microbiome and their Potential Metabolites Analysis Methodology

3.17.1 Data Collection and Preparation

The data of ASD related metabolites has been extracted using literature mining with the help of The Human Metabolome Database (HMDB) (https://hmdb.ca/). The whole data is compiled and organized into an Excel file which contains all the necessary details required for further metabolome analysis.

Through Virtual Metabolic Human (VMH) Database Search (https://www.vmh.life / resources/ intro/index.html), different bacterial species related to autism-related metabolites are identified and downloaded.

This data is also organized and compiled into an Excel file in the form of name of genus, name of species, related health condition, microbial response/effect, metabolite ID, metabolite name, class, revised genus, and metabolic pathway.

3.17.2 Data Retrieval using Python

The downloaded data from the Human Metabolome Database (HMDB) and Virtual Metabolic Human (VMH) Database Search contain files corresponding to metabolites and metabolic reactions data for each identified bacterial species. Then we employed a keyword search (keywords from the lists we curated) on these "Metabolites" and "Reactions" lists through Python script using Panda library for further analysis. The Python script for this analysis is as follows:

Python Code for Metabolite Data Processing

```
# Import essential libraries for data processing
import pandas as pd

# Define the path to your file
file_path = '/content/Metabolites.xlsx'   # Update this with the
actual file path
```

```
    # Load the file into pandas DataFrame
    df = pd.read_excel(file_path)   # Adjust separator if needed

    # Display the column names in the DataFrame
    print("Columns in the dataFrame:")
    print(df.columns)

    # Display the first few rows of the data frame
    df.head()

    # List of known autism-related metabolites
    autism_related_keywords = [
'p-cresol', 'Glutathione', 'Cysteine', 'Carnitine', 'Taurine', '
 Hypotaurine',
 'Melatonin', 'Lactate', 'Branched-Chain   Amino Acids', 'Valine',
 'Leucine',
 'Isoleucine', 'Serotonin', 'Hippurate', 'Kynurenic Acid', '
Quinolinic Acid',
 'Folate', 'Vitamin B6', 'Vitamin B12', 'Short-Chain Fatty Acids',
 'Acetate',
 'Propionate', 'Butyrate', 'Propiomazine', '3-hydroxy-3-(3-
hydroxyphenyl)propanoic acid-O-sulphate',
 'Gliadorphin', 'Gluten exorphin B5', 'Gluten exorphin C', 'Gluten
 exorphin B4',
 'Gluten exorphin A5', '11Z-Eicosenoic acid', '2-Pentylthiophene',
 'Casomorphin',
 'Ethyl propionate', '4-Heptanone', 'Iminodiacetic acid', '3-
Hexanone',
 'Dimethylethanolamine', '2-Phenylethanol', 'Toluene', '
Adenylsuccinic acid',
 'Melatonin', 'Benzene', 'Nonanal', 'Oxalic acid', '2-
Methylbutyrylglycine',
 'Indolylacryloylglycine', 'Propionic acid', 'Acetylcysteine', '
Mercury'
 ]
 # Function to identify if any autism-related metabolites are in
the full name
 def contains_autism_metabolite(fullName):
```

```
 return any(metabolite.lower() in fullName.lower() for metabolite
in autism_related_keywords)
 # Apply the function to filter the dataframe
 autism_related_metabolites_df = df[df['fullName'].apply(
contains_autism_metabolite)]

 # Display the autism-related metabolites
 autism_related_metabolites_df

 # Save the filtered results to a new file
 autism_related_metabolites_df.to_csv('autism_related_metabolites.
csv', index=False)

 # Define the path to a file
 file_path = '/content/Reactions.xlsx'   # Update this with the
actual file path

 # Load the file into pandas DataFrame
 df = pd.read_excel(file_path)   # Adjust separator if needed

 # Display the column names in the DataFrame
 print("Columns in the dataFrame:")
 print(df.columns)

 # Display the first few rows of the data frame
 df.head()

 # List of known autism-related metabolites
 autism_related_keywords = [
 'p-cresol', 'Glutathione', 'Cysteine', 'Carnitine', 'Taurine', '
Hypotaurine',
 'Melatonin', 'Lactate', 'Branched-Chain Amino Acids', 'Valine', '
Leucine',
 'Isoleucine', 'Serotonin', 'Hippurate', 'Kynurenic Acid', '
Quinolinic Acid',
 'Folate', 'Vitamin B6', 'Vitamin B12', 'Short-Chain Fatty Acids',
 'Acetate',
```

```
 'Propionate', 'Butyrate', 'Propiomazine', '3-hydroxy-3-(3-
hydroxyphenyl)propanoic acid-O-sulphate',
 'Gliadorphin', 'Gluten exorphin B5', 'Gluten exorphin C', 'Gluten
 exorphin B4',
 'Gluten exorphin A5', '11Z-Eicosenoic acid', '2-Pentylthiophene',
 'Casomorphin',
 'Ethyl propionate', '4-Heptanone', 'Iminodiacetic acid', '3-
Hexanone',
 'Dimethylethanolamine', '2-Phenylethanol', 'Toluene', '
Adenylsuccinic acid',
 'Melatonin', 'Benzene', 'Nonanal', 'Oxalic acid', '2-
Methylbutyrylglycine',
 'Indolylacryloylglycine', 'Propionic acid', 'Acetylcysteine', '
Mercury'
 ]

 # Create a pattern to search for these keywords in the
description
 pattern = '|'.join(autism_related_keywords)

 # Filter the DataFrame for rows containing autism-related
keywords in the 'description' column
 autism_related_reactions = df[df['description'].str.contains(
pattern, case=False, na=False)]

 # Display the filtered DataFrame
 print("Autism-related reactions:")
 print(autism_related_reactions)

 # Save the filtered results to a CSV file
 autism_related_reactions.to_csv('autism_related_reactions.csv',
index=False)
 print("Autism-related reactions have been saved to '
autism_related_reactions.csv'")
```

3.17.3 Metabolic Pathway Analysis

Pathway and enrichment analyses are central component of metabolomic data interpretation, as they link metabolite changes to biological processes, pathways and disease mechanisms. For this purpose, Metaboanalyst (https://www. metaboanalyst .ca/) web-based platform is used which revolves around the integration of advanced statistical, machine learning and pathway analysis tools to provide comprehensive insights into metabolomic dataset.

The enrichment analysis through metaboanalyst provides insights into higher-level biological functions and their disease mechanisms. Microbial metabolites play a significant role in various diseases, and their analysis can provide insights into disease mechanisms and potential therapeutic targets. It provides a comprehensive database of metabolic pathways that can be used to analyze the interactions between microbial metabolites and human diseases. Its key steps includes:

FIGURE 3.9: Metaboanalyst pathway analysis for Autism related metabolites.

3.17.3.1 Mapping Input Metabolites to Pathways

The input metabolites are matched with compounds in curated databases using their unique identifiers (e.g., KEGG ID, HMDB ID, PubChem ID). This mapping is essential to align the experimental data with reference pathways.

3.17.3.2 Pathway Scoring

Over-Representation Analysis (ORA): Tests whether a particular pathway is overrepresented in the dataset by comparing the observed number of metabolites in a pathway to what would be expected by chance. Uses statistical tests (e.g.,

hypergeometric test) to calculate p-values, identifying pathways with significantly altered activity.

Topological Analysis: Examines the position of metabolites within the pathway structure to account for their importance (e.g., key intermediates or terminal nodes). Metabolic nodes with higher connectivity or central roles (e.g., hubs) are weighted more significantly.

3.17.3.3 Pathway Prioritization

Pathways are ranked based on their biological relevance, combining, Statistical significance from ORA and Topological importance from network structure.

3.17.3.4 Visualization

Pathways are often represented as interactive graphs or maps, highlighting the metabolites detected in the data.

Chapter 4

Results

The current study was conducted in order to explore the etiology of ASD by deeply studying the mircobiota-gut-brain axis. The study encompassed the meta-analysis to draw the consistent results about variations in gut microbiota. It was followed by meta-genome analysis so that gut microbial alterations could be figured out in ASD subjects as opposed to controls. Finally, the possible mechanism through which gut microbiota could affect brain physiology was figured out through metabolites produced by gut microbiota in human gut.

4.1 Meta-analysis (RQ 1)

Pertaining to objective no. 1, the literature search was carried out to collect the relevant records related to the alterations or associations of gut microbial composition in ASD individuals. and resulted in 2596 records that were found to be potentially relevant. Titles and abstracts of these records were carefully scrutinized, duplicates from various databases were removed and a total 148 articles were further selected, and their full texts were completely studied. After exclusion of studies that were based on non-human subjects, reviews, and lacking association between the studies, 57 studies were further selected. Due to non-availability of required data in terms of bacterial abundance or percentage, 36 studies were excluded and 21 studies were

finally selected as fully eligible for this meta-analysis. The selection procedure is shown in figure 4.1.

4.1.1 Inclusion and Exclusion Criteria

4.1.1.1 Inclusion Criteria

The inclusion criteria for the meta-analysis is as follows:

Only observational studies, including case-control and cohort studies conducted on human populations, were considered. Articles published from the inception of relevant research up to 31st July 2023 were included. The studies focused on children, with an age limit of up to 18 years for the participants were under investigation. Only those articles that provided the comparative data on the gut microbiota (GM) between children with autism and their age-matched controls were considered.

Additionally, studies that explicitly reported the significant differences in the relative abundance of gut microbiota between these groups were included. The researches that utilized advanced sequencing techniques, such as 16S rRNA sequencing or whole metagenome sequencing, to profile the gut microbiome were considered. Furthermore, only articles written in the English language were eligible for inclusion in this meta-analysis.

4.1.1.2 Exclusion Criteria

The exclusion criteria for meta-analysis is as follows: Studies conducted in vitro or involving non-human subjects were excluded. Systematic reviews and meta-analyses, as well as book chapters, editorial pages, and dissertations, were also excluded from consideration. Those types of publications which do not meet the focus of original research on human populations or the specific research methodologies outlined in the inclusion criteria were also excluded. The databases quested to conduct the meta-analysis were Scopus, Web of Science, PubMed, Cochrane databases, and Science

database. The chosen database use peer-reviewed articles, with the data set and the experimental evaluation are were verified. We used research published in reputed journals.

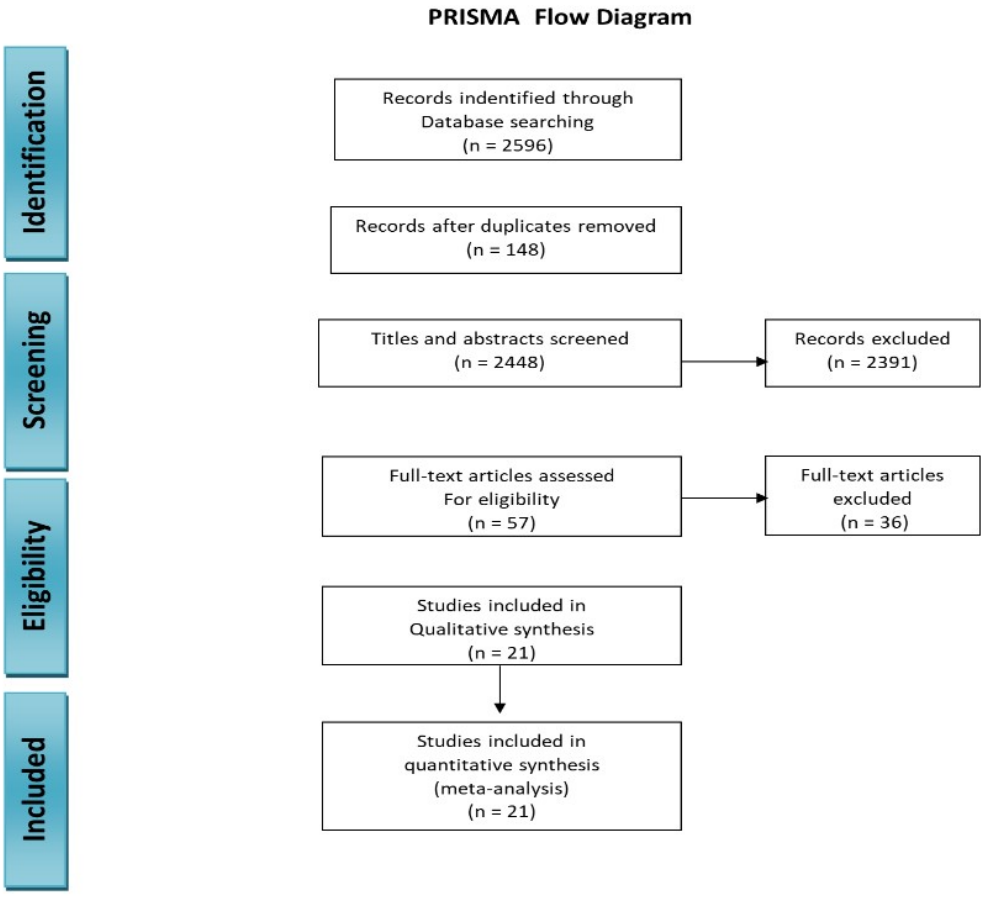

FIGURE 4.1: PRISMA flow diagram for the data search and the included studies.

4.1.2 Characteristics of Included Articles

Characteristics of the studies conducted from 2005 to July 2023. Most of the included studies were from China (seven) [99, 101, 106, 111, 117, 121, 122], four from United States of America [81, 87, 93, 98], three from Australia [82, 85, 112], three from Italy [88, 94, 151], one each from Slovakia [89], Japan [90], Korea [96], and Spain [104]. The sample size was from 6 to 138, number of ASD subjects were 773 and control children were 629, and the ages ranged between 2 to 17 years. The study design of six studies was cohort based, whereas rest of the studies did not declare the study design.

TABLE 4.1: Demographic characteristics of study participants

Sr. No.	Name of Author	Year	Population	Study Design
1	Finegold et al.,	2010	USA	ND
2	Adams et al.,	2011	USA	ND
3	Wang et al.,	2011	Australia	Cohort
4	Gondalia et al.,	2012	Austrailia	ND
5	Angelis et al.,	2013	Italy	ND
6	Kang et al.,	2013	USA	ND
7	Tomova et al.,	2015	Slovakia	ND
8	Inoue et al.,	2016	Japan	ND
9	Kang et al.,	2017	USA	Cohort
10	Lee et al.,	2017	Korea	ND
11	Strati et al.,	2017	Italy	Cohort
12	Coretti et al.,	2018	Italy	Cohort
13	Zhang et al.,	2018	China	ND
14	Ma et al.,	2019	China	ND
15	Plaza-Diaz et al.,	2019	Spain	Cohort
16	Sun et al.,	2019	China	ND
17	Shili et al.,	2021	Austrailia	Cohort
18	Ding et al.,	2021	China	ND
19	Zilin et al.,	2021	China	ND
20	Wong et al.,	2022	China	ND
21	Hong et al.,	2022	China	ND

Most of the studies employed 16S rRNA gene sequencing to analyze the differences in the gut microbiota, two studies were investigated on culture-based methods, and two studies employed Polymerase Chain Reaction (PCR), and stool sample was taken for analysis in all studies. The 16S rRNA gene sequencing is a widely used method to assess the gut bacterial composition. The choice of optimal variable regions might vary depending on the analysis target, the specificity of the primers, the GC contents

in the selected region, and the bacterial compositions of various samples.

TABLE 4.2: Details of patients vs controls from each study

Sr. No.	**ASD (n)**	**Gender**		**Age (years)**	**Neuro-typical**	**Gender**		**Age (years)**
		M	**F**			**M**	**F**	
1	33	24	9	2–13	8	5	3	2–13
2	58	50	8	6.91 ± 3.4	39	18	21	7.7 ± 4.4
3	23	21	2	10.25± 0.75	9	4	5	9.5± 1.25
4	51	42	9	2–12	53	19	34	2–12
5	10	ND	ND	4–10	10	ND	ND	4-10
6	20	17	3	8.3±4.4	20	18	2	6.7±2.7
7	10	9	1	2-9	10	10	0	2-11
8	6	ND	ND	3-5	6	ND	ND	3-5
9	23	22	1	4-17	21	16	6	4-17
10	20	18	2	22.4±4.9	28	24	4	21.1±9.5
11	40	31	9	5-17	40	28	12	5-17
12	11	9	2	35 ± 5.7	14	8	6	35 ± 8.4
13	35	29	6	4.9±1.5	6	5	1	4.6±1.1
14	45	39	6	7.04 ± 1.19	45	39	6	7.27 ± 1.07
15	48	ND	ND	43.69 ± 2.7	57	ND	ND	51.00 ± 2.59
16	9	8	1	3-12	6	4	2	3-12
17	40	31	9	11.1 ± 6.8	40	28	12	9.2 ± 7.9
18	25	21	4	5.7 ± 1.4	20	12	8	5.4 ± 1.8
19	138	117	21	6.11 ± 2.00	60	27	33	6.65 ± 2.22
20	92	30	62	8.2	112	32	80	8.469
21	36	30	6	3.86 ± 2.22	25	20	5	4.12 ± 1.83

Microbiota was mainly studied at phylum and genus level, and a wide range of microbes were reported in terms of percentage. All ASD subjects of included studies were reported having GIS, except 4 studies where co-morbidity was not declared.

TABLE 4.3: Details of microbiota variartion from each Study

Sr.	**Detail of Microbiota**			**Diagnosis**	**Tool**	**Com-**
No.	**Assesment**	**Outcome**	**Unit**			**orbity**
1	16S rRNA gene sequencing	**Phylum:** Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Cyanobacteria, Fusobacteria, Verrucomicrobia, Tenericutes **Genus:** *Akkermansia, Bacteroides, Bifidobacterium, Clostridium, Faecalibacterium, Parabacteroides, Ruminococcus*	R.A	ASD	DSM-V	GIS
2	Culture based methods	**Genus:** *Lactobacillus, Bifidobacterium, Enterococcus*	CFU	Autism, PDD/, NOS or Aspergers	(ATEC)	GIS
3	qPCR	**Genus:** *Akkermansia, Bacteroides, Bifidobacterium, Faecalibacterium, Clostridium*	R.A	Autistic disorder, Asperger's syndrome, Regressive autism	DSM IV and	FGID

4	pyro sequencing	**Phylum:** Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Cyanobacteria, Fusobacteria, Verrucomicrobia, Tenericutes Genus: Anaerostipes, Anaerotruncus, Bacteroides, Bifidobacterium, Blautia, Clostridium, Faecalibacterium, Parabacteroides, Ruminococcus, Sutterella, Veillonella, Coprococcus, Dialister, Dorea, Roseburia, Phascolarcto-bacterium	R.A	ASD Asperger's syndrome,	Quest-ionnaire CARS	ND
5	Culture based methods	**Genus:** *Akkermansia, Bacteroides, Bifidobacterium,*	R.A	PDD-NOS, AD	DSM-IV-TR, ADI-R, ADOS	ND

	Clostridium,			and	
	Faecalibacterium,			CARS	
	Parabacteroides,				
	Ruminococcus				
6	**Phylum:**	R.A	ASD	ADI-	GIS
	Bacteroidetes			Revised,	
	Firmicutes			ADOS,	
	Proteobacteria			ATEC,	
	Actinobacteria			PDD-BI	
	Cyanobacteria,				
	Fusobacteria				
	Verrucomicrobia				
	Tenericutes				
	Genus:				
	Akkermansia				
	Anaerostipes				
	Anaerotruncus				
	Bacteroides				
	Bifidobacterium				
	Blautia				
	Clostridium				
	Faecalibacterium				
	Parabacteroides				
	Ruminococcus				
	Sutterella				
	Veillonella				
	Coprococcus				
	Dialister				
	Dorea				
	Phascolarcto-				
	bacterium				

		Roseburia				
7	Real-time PCR	**Phylum:** Bacteroidetes Firmicutes	R.A	ASD	ICD-10 CARS, ADI	GIS
8	16S rRNA gene sequencing pyroseq-uencing	**Genus:** *Akkermansia Anaerostipes Anaerotruncus Bacteroides Bifidobacterium Blautia, Clostridium Faecalibacterium Parabacteroides Ruminococcus Veillonella Coprococcus Dialister Dorea, Phascolarcto-bacterium Roseburia Sutterella*	R.A	ASD	DSM-V	ND
9	16S rRNA amplicon gene sequencing	**Genus:** *Akkermansia Anaerostipes, Bacteroides Faecalibacterium, Coprococcus Roseburia*	R.A	ASD	ATEC, PDD-BI	GIS
10	16S rRNA	**Phylum:**	R.A	ASD	DSM-V,	GIS

	gene sequencing	Firmicutes Verrucomicrobia Proteobacteria, Cyanobacteria **Genus:** *Streptococcus* *Akkermansia* *Jeotgalicoccus* *Desulfovibrio* *Oscillospira* *Rhodococcus* *alomonas* *Pseudomonas* *Sphingomonas* *Agrobacterium* *Achromobacter* *Roseateles*			CARS	
11	16S rRNA gene sequencing	**Phylum:** Bacteroidetes Firmicutes Proteobacteria Actinobacteria Cyanobacteria Fusobacteria Verrucomicrobia Genus: Akkermansia Anaerostipes Anaerotruncus Bacteroides Bifidobacterium	R.A	ASD	DSM-V, CARS	GIS

		Blautia Clostridium Faecalibacterium Dorea Parabacteroides Ruminococcus Sutterella Veillonella Coprococcus Dialister Phascolarcto- bacterium Roseburia				
12	16S rRNA gene sequencing	**Phylum:** Bacteroidetes Firmicutes Proteobacteria Actinobacteria **Genus:** *Bacteroides* *Bifidobacterium* *Blautia* *Faecalibacterium* *Parabacteroides* *Ruminococcus* *Coprococcus* *Roseburia*	R.A	ASD	DSM V, ADI-R, ADOS 2, CARS	GIS
13	16S rRNA gene sequencing	**Phylum:** Bacteroidetes Genus: Veillonella	R.A	ASD	DSM-V	GIS

		Streptococcus Eschericia				
14	16S rRNA gene sequencing	**Phylum:** Bacteroidetes Firmicutes Proteobacteria Actinobacteria Cyanobacteria Fusobacteria Verrucomicrobia Tenericutes **Genus:** Bacteroides Bifidobacterium Blautia, Clostridium Faecalibacterium, Ruminococcus Coprococcus Phascolarcto- bacterium Roseburia Parabacteroides	R.A	ASD	DSM V, CARS	GIS
15	16S rRNA amplicon gene sequencing	**Phylum:** Bacteroidetes Firmicutes Proteobacteria Actinobacteria Verrucomicrobia **Genus:** *Akkermansia*	R.A	ASD	DSM V, ADI-R, PDDBI	GIS

		Bacteroides *Bifidobacterium,* *Clostridium,* *Faecalibacterium* *Parabacteroides* *Ruminococcus* *Veillonella*				
16	16S rRNA gene sequencing	**Genus:** *Prevotella*	R.A	ASD	DSM-V, CARS	ND
17	16S rRNA gene sequencing	**Phylum:** Firmicutes Bacteroidetes	R.A	ASD	DSM-V, CARS	GIS
18	16S rRNA gene sequencing	**Phylum:** Firmicutes Actinobacteria **Genus:** *Faecalibacterium* *Prevotella* *Subdoligranulum* *Ruminococcus* *Bifidobacterium*	R.A	ASD	ATEC, DSM-V	GIS
19	16S rRNA gene sequencing	**Genus:** *Bacteroides* *Faecalibacterium* *Sutterella* *Collinsella* *Prevotella* *Coprococcus* *Desulfovibrio*	R.A	ASD	CARS, ADOS, DSM-V	GIS
20	16S rRNA	**Phylum:**	R.A	ASD	DSM-V,	FGID

	gene sequencing	Firmicutes Bacteroidetes **Genus:** *Bifidobacterium* *Dorea* *Blautia* *Collinsella* *Bacteroides* *Alistipes* *Parabacteroides* *Sutterella*			CARS	
21	16S rRNA gene sequencing	**Genus:** *Bifidobacterium*	R.A	ASD	DSMMD, ABC, CARS	ND

Note: All studies from S.No. 1-21 analysed stool samples except study from study (S.No. 10) analysed urine samples

The RA or percentage of bacteria was used to conduct this meta-analysis and construct the forest plots. The cumulative results of the present meta-analysis are shown in table 4.4 and 4.5.

TABLE 4.4: Phylum and genus level results of meta-analysis

Sr.	**Microbes**	**No.**	**ASD**			**Control**		
No.		**Sty**	**RA**	**95% CI**	**I^2**	**RA**	**95% CI**	**I^2**
1	*Bacteroidetes*	4	1.14	0.38-1.90	89	0.92	0.21-1.62	97
2	*Firmicutes*	7	0.43	0.19-0.67	98	0.33	0.18-0.47	97
3	*Bacteroides*	13	12.5	10.1-14.9	99	9.84	8.22-11.46	99
4	*Faecalibacterium*	12	5.75	4.56-6.93	99	1.93	1.44-2.43	99
5	*Clostridium*	9	1.91	1.34-2.48	96	0.32	0.10-0.53	96

6	*Phascolarcto-bacterium*	5	0.17	0.07-0.26	64	0.12	0.02-0.22	91
7	*Bifidobacterium*	12	0.79	0.57-1.01	99	1.74	1.38-2.09	100
8	*Parabacteroides*	10	0.75	0.41-1.10	95	0.97	0.40-1.55	95
9	*Coprococcus*	9	0.13	0.07-0.18	97	0.29	0.18-0.40	97
10	*Prevotella*	6	0.17	0.02-0.32	87	0.34	0.09-0.60	93

Note: RA "relative abundance", CI "confidence interval", Z "overall effect size"

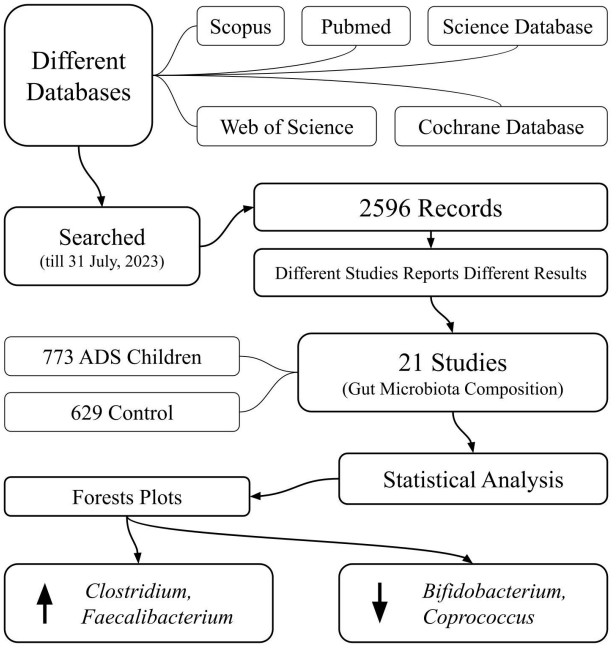

FIGURE 4.2: Pictorial representation of meta-analysis conducted in the current study and results obtained through it.

TABLE 4.5: Phylum and genus level results of meta-analysis (microbial variation).

Sr. No	**Microbes**	**Overall Effect**		**Subgroup Differences**		**Diff.**
		Z	**P**	**I^2**	**P**	**%**
1	*Bacteroidetes*	3.38	<0.001	0	0.67	1.23

2	*Firmicutes*	7.61	<0.001	0	0.48	1.3
3	*Bacteroides*	18.27	<0.001	69	0.07	1.27
4	*Faecalibacterium*	12.63	<0.001	97	<0.001	2.98
5	*Clostridium*	5.59	<0.001	96.2	<0.001	5.97
6	*Phascolarcto bacterium*	3.6	<0.001	0	0.53	1.42
7	*Bifidobacterium*	11.85	<0.001	95	<0.001	0.45
8	*Parabacteroides*	10.41	<0.001	0	0.52	0.77
9	*Coprococcus*	7.93	<0.001	84.9	0.01	0.45
10	*Prevotella*	4.64	<0.001	23.8	0.25	0.5

4.1.3 More Abundant Bacterial Phyla in ASD

4.1.3.1 *Bacteroidetes*

The meta-analysis of Bacteroidetes encompassed four studies, and showed the results of 1.14% (95%CI: 0.38, 1.90) in ASD subjects and 0.92% (95% CI: 0.21, 1.62) in the control group. For ASD subjects and the control group, the heterogeneity was high (I^2= 89%, and I^2=97% respectively) for the included studies, and the subgroups heterogeneity was 0%. The overall effect size was moderate and significant (Z= 3.38, p¡0.001). As the subgroup heterogeneity was zero, and p value was > 0.05, the results obtained are non-significant. The forest plot is given in figure 4.3.

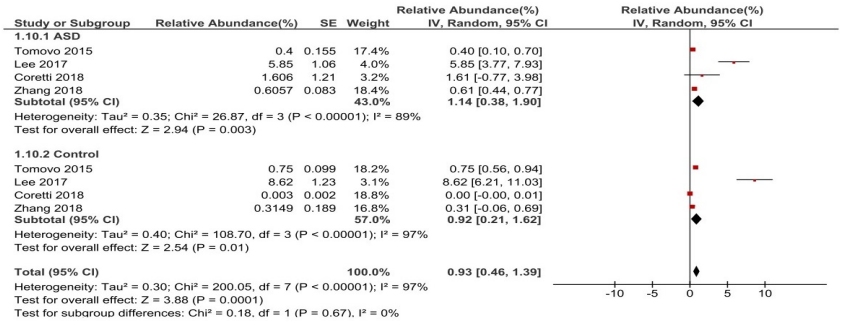

FIGURE 4.3: Forest plot for *Bacteroidetes*, showing the higher abundance of the genus in ASD subjects as compared to the control group.

4.1.3.2 *Fermicutes*

Our random-effect meta-analysis included seven studies with 0.43% (95% CI:0.19, 0.67) in ASD subjects and 0.33% (95% CI: 0.18, 0.47) in neurotypical children. Between-studies heterogeneity was 98% and 97% for ASD and control group respectively, and zero% when the both groups were compared. The overall effect size was found to be 7.61, which is quite large, and also significant ($p<0.001$). However, I^2 between the two groups was zero and p value was > 0.05, the results are considered as non-significant. The forest plot is given in figure 4.4.

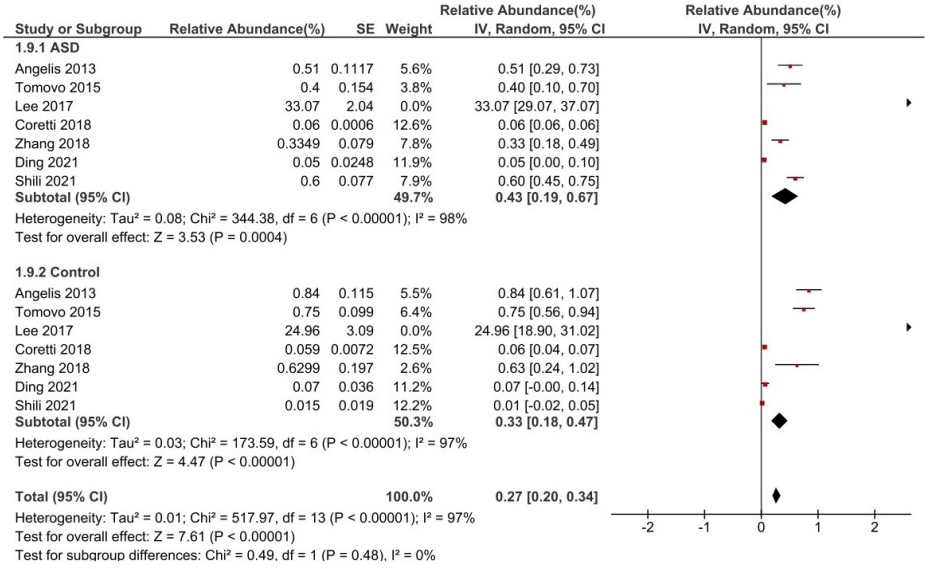

FIGURE 4.4: Forest plot for *Fermicutes*, showing the higher abundance of the genus in ASD subjects as compared to the control group.

4.1.4 More Abundant Bacterial Genera in ASD

4.1.4.1 *Bacteroides*

In order to calculate the RA of Bacteroides, thirteen studies were included. The level of Bacteroides was found to be 12.48% (with a 95% CI of 10.10, 14.85) in ASD and 9.84% (8.22, 11.46) in control subjects. In both of the groups, a high heterogeneity (I^2= 99%) was found between the included studies, and the heterogeneity between

the subgroups was 69.0%. The overall effect size was quite large (Z=18.27%) and significant (p<0.001).

Both groups depicted a difference of 1.27 in bacterial percentage, implying that the ASD subjects showed a higher percentage of Bacteroides as compared to the controls. However, non-significant p value of the subgroup heterogeneity obscure the results. Forest plot for *Bacteroides*, showing the higher abundance of the genus in ASD subjects as compared to the control group is given in figure 4.5.

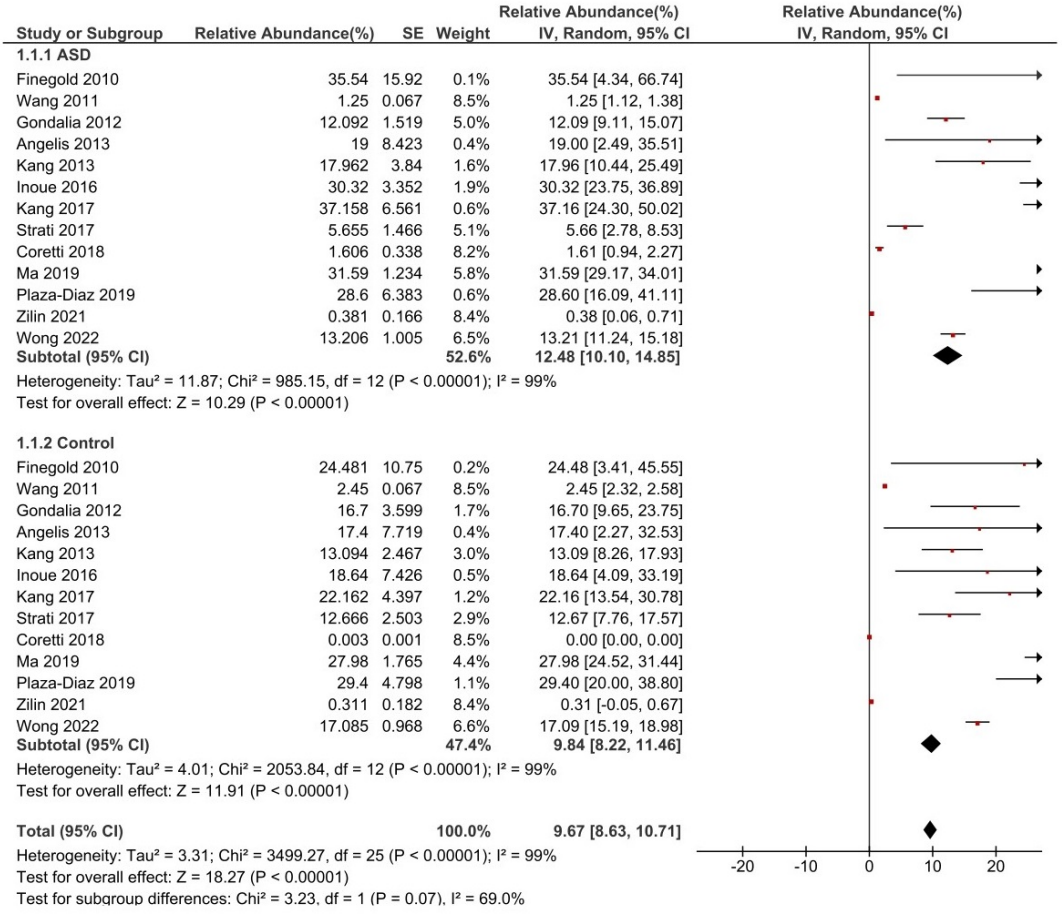

FIGURE 4.5: Forest plot for *Bacteroides*, showing the higher abundance of the genus in ASD subjects as compared to the control group.

4.1.4.2 *Faecalibacterium*

To conduct the meta-analysis for *Faecalibacterium*, a set of twelve studies was taken in. ASD subjects showed a percentage of 5.75% (95% CI: 4.56, 6.93) with respect

to *Faecalibacterium* whereas the control group depicted 1.93% (95% CI: 1.44, 2.43) of Faecalibaterium. A very high heterogeneity (I^2= 99%) was found between the studies for ASD and control group, and the subgroup heterogeneity was also high (I^2= 97%). A quite large and significant effect size was found (Z=12.63, p<0.001). ASD and control group showed a difference of 2.98 in bacterial percentage, explicitly showing the higher abundance of *Faecalibacterium* in ASD subjects than in controls. Statistically significant results are obtained for the differences of this genus. The forest plot constructed for this genus is shown in figure 4.6.

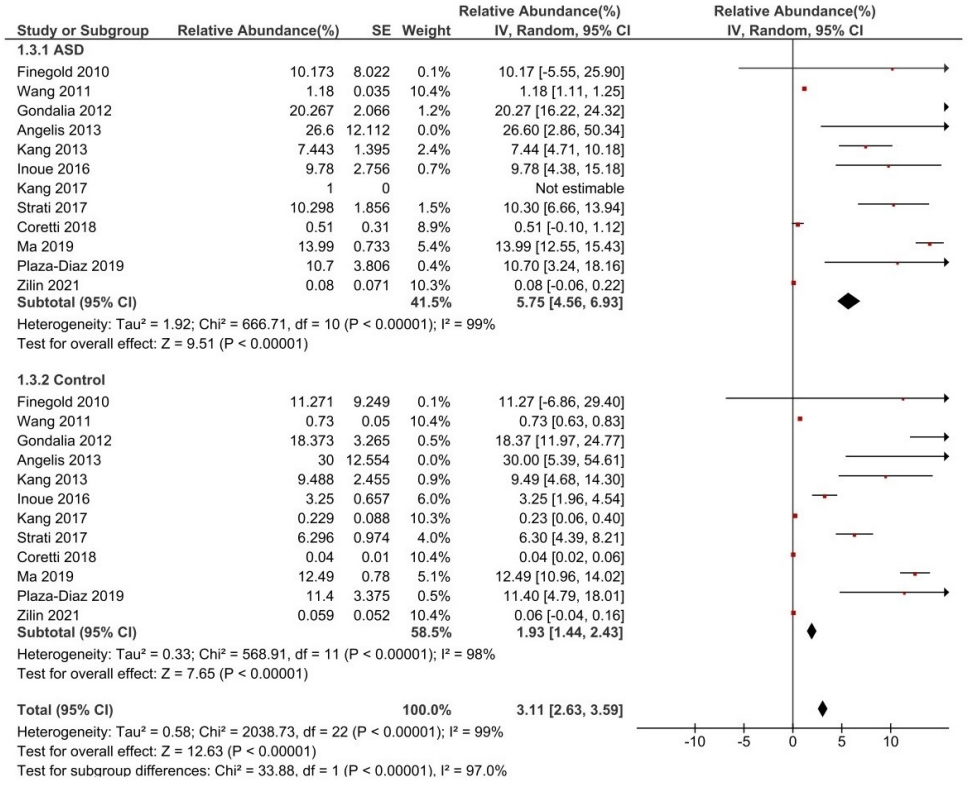

FIGURE 4.6: Forest plot for *Faecalibacterium*, showing the higher abundance of the genus in ASD subjects as compared to the control group.

4.1.4.3 *Clostridium*

The random-effect meta-analysis incorporated nine studies. *Clostridium* showed percentages of 1.91% (95% CI: 1.34, 2.48) in ASD subjects and 0.32 (95% CI: 0.10, 0.53)in control group. Both groups showed a high heterogeneity (I^2=96%) between

the included studies, and the subgroup heterogeneity was also found to be high (I^2= 96.2%).

The overall effect size was large as well as significant (Z=5.59, p¡0.001). The difference of 5.97 in bacterial percentage was found between the both group, clearly demonstrating that the ASD subjects show a higher percentage of *Clostridium* as compared to the control group. The results obtained are statistically significant, and the forest plot is shown in figure 4.7.

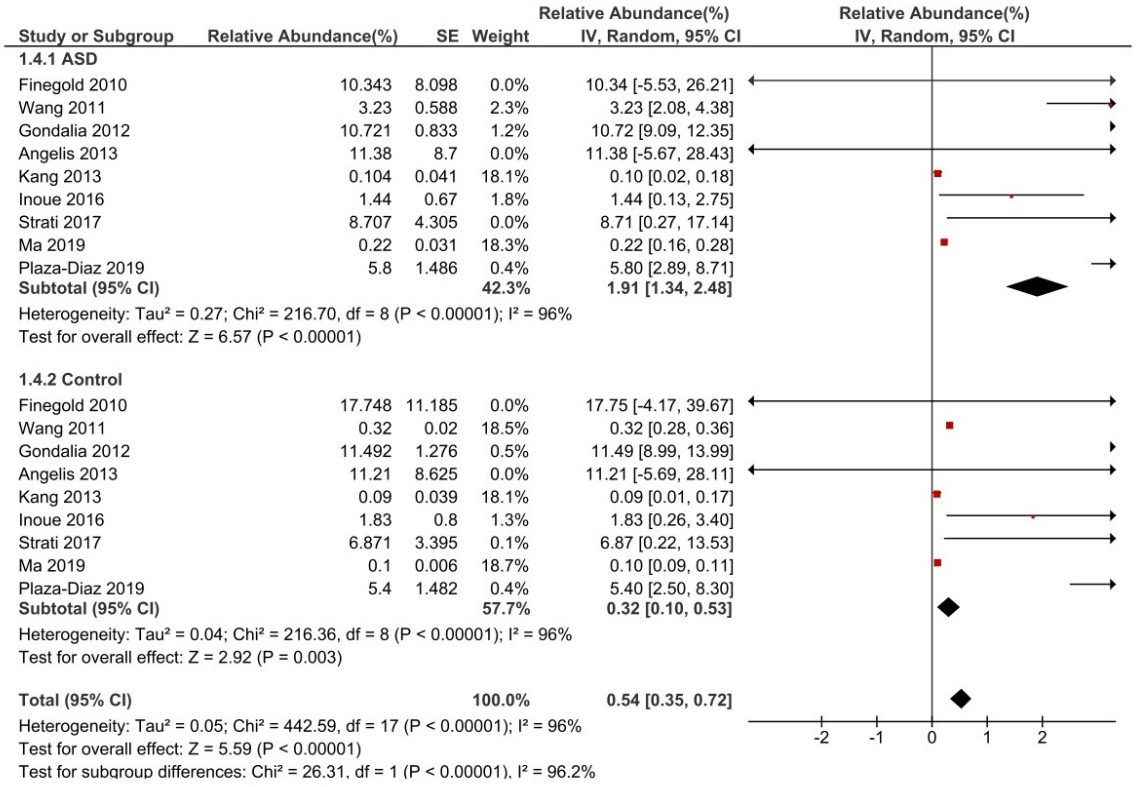

FIGURE 4.7: Forest plot for *Clostridium*, with the conclusions of higher relative abundance in ASD group as compared to the control group.

4.1.4.4 *Phascolaractobacterium*

The meta-analysis involved five trials for *Phascolarctobacterium* and resulted in 0.17% (95% CI: 0.07, 0.26) in ASD subjects and 0.12% (95% CI: 0.02, 0.22) in control group.

Between-study heterogeneity was 64% for ASD group and 91% for control group. The comparison showed heterogeneity to be zero% between the two groups. The overall effect size was concluded to be moderate (Z=3.60) and statistically significant (p<0.001).

A difference 1.42 in bacterial percentages was found between the ASD and control group, showing that the ASD group had higher abundance of *Phascolarctobacterium* as compared to the control group.

Between the group heterogeneity was found to be zero, and p value was >0.05, thus resulting in non-significant statistics. The forest plot is given in figure 4.8.

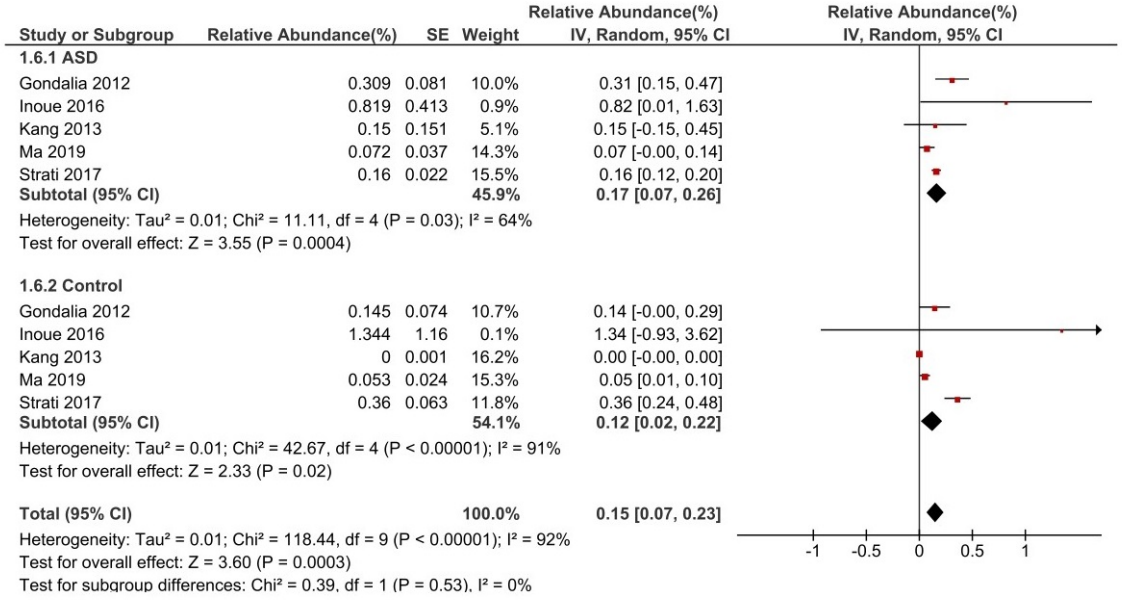

FIGURE 4.8: Forest plot for *Phascolarctobacterium* in ASD

4.1.5 Less Abundant Bacterial Genera in ASD

4.1.5.1 *Bifidobacterium*

Our meta-analysis included twelve trials for *Bifidobacterium*, depicting the results to be 0.79% (95% CI: 0.57, 1.01) in the ASD group and 1.74% (95% CI: 1.38, 2.09) in the neurotypical group. ASD subjects and the control showed a higher heterogeneity (I^2 =

99% and 100%, respectively) between the included studies. A quite high heterogeneity ($I^2 = 100\%$) was found by comparing the two groups. The values of overall effect size were found to be quite large ($Z = 11.85$) and highly significant ($p < 0.001$). The bacterial percentage delineated a difference of 0.45, indicating a lower level of *Bifidobacterium* in the ASD group compared to the neurotypical group.

Statistically significant results were obtained for this genus, suggesting a potential role of *Bifidobacterium* in ASD-related gut dysbiosis. The findings align with previous studies indicating altered gut microbiota composition in ASD individuals. However, variations in sequencing techniques and study populations may contribute to the observed heterogeneity. Further investigations with standardized methodologies are needed to confirm these associations. The forest plot constructed is shown in figure 4.9.

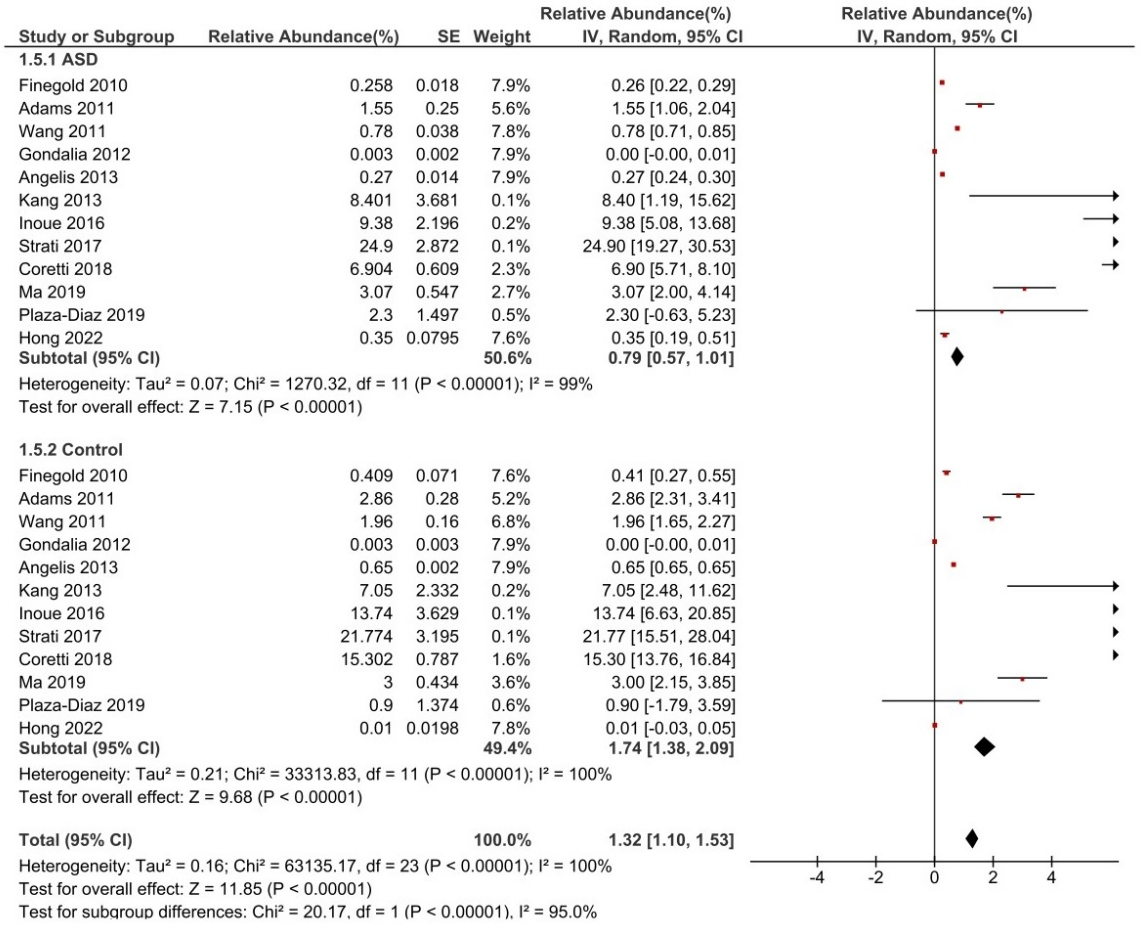

FIGURE 4.9: Forest plot for *Bifidobacterium* that shows the lower abundance of the genus in ASD subjects as opposed to the control group.

4.1.5.2 *Parabacteroides*

Ten studies were involved in the random-effect meta-analysis of *Parabacteroides*. The outcome indicated that the level of this genus in ASD group was 0.75% (95% CI: 0.41, 1.10) whereas in the control group, it was 0.97% (95% CI: 0.40, 1.55). A very high heterogeneity of 95% (in both of the groups) was found for the included studies. The subgroup heterogeneity was zero%. The overall effect size was 10.41 (quite large) and found to statistically significant ($p<0.001$). *Parabacteroides* was found to be less abundant in ASD group than the control group owing to the difference of 0.77 in bacterial percentages. As the subgroup heterogeneity was zero%, and p value was $>$ 0.05, the results obtained are not considered as significant. The forest plot is given in figure 4.10.

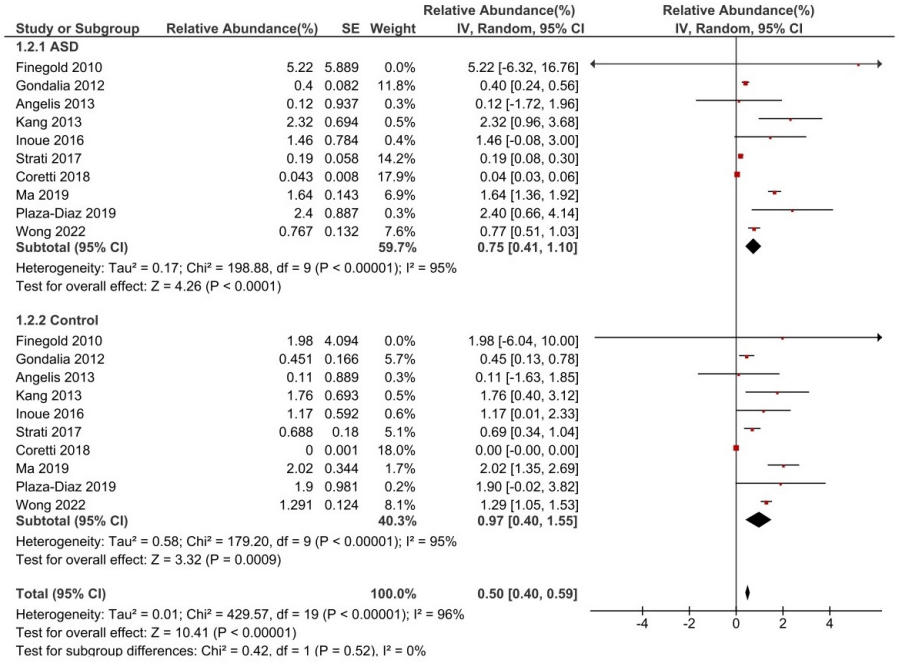

FIGURE 4.10: Forest plot for *Parabacteroides* , depicting the lower relative abundance of this genus in ASD subjects as compared to the control group.

4.1.5.3 *Coprococcus*

In an endeavor to carry out the meta-analysis for *Coprococcus*, nine trials were involved. The percentage of this genus was found to be 0.13% (95% CI: 0.07, 0.18) in

the ASD group as opposed to 0.29% (95% CI: 0.18, 0.40) in the control group. The included studies showed a high heterogeneity (I^2 = 97%) for the ASD and control groups, and a higher heterogeneity (I^2 = 84.9%) between the subgroups as well. The overall effect size was summarized to be large (Z = 7.93) and significant ($p < 0.001$). The ASD group and the control group delineated a difference of 0.45 in bacterial percentage, thereby showing a lower abundance of this genus in ASD subjects than in the control group. The obtained results are statistically significant, suggesting a potential association between *Coprococcus* abundance and ASD. Since *Coprococcus* is known for its role in short-chain fatty acid (SCFA) production, its reduced levels in ASD individuals may indicate potential disruptions in gut metabolic pathways. These findings support previous research linking gut microbiota imbalances to neurodevelopmental disorders. However, variations in study methodologies, sequencing platforms, and sample sizes may contribute to the observed heterogeneity. Further large-scale studies with standardized protocols are necessary to validate these associations and explore their clinical implications. The forest plot is shown in figure 4.11.

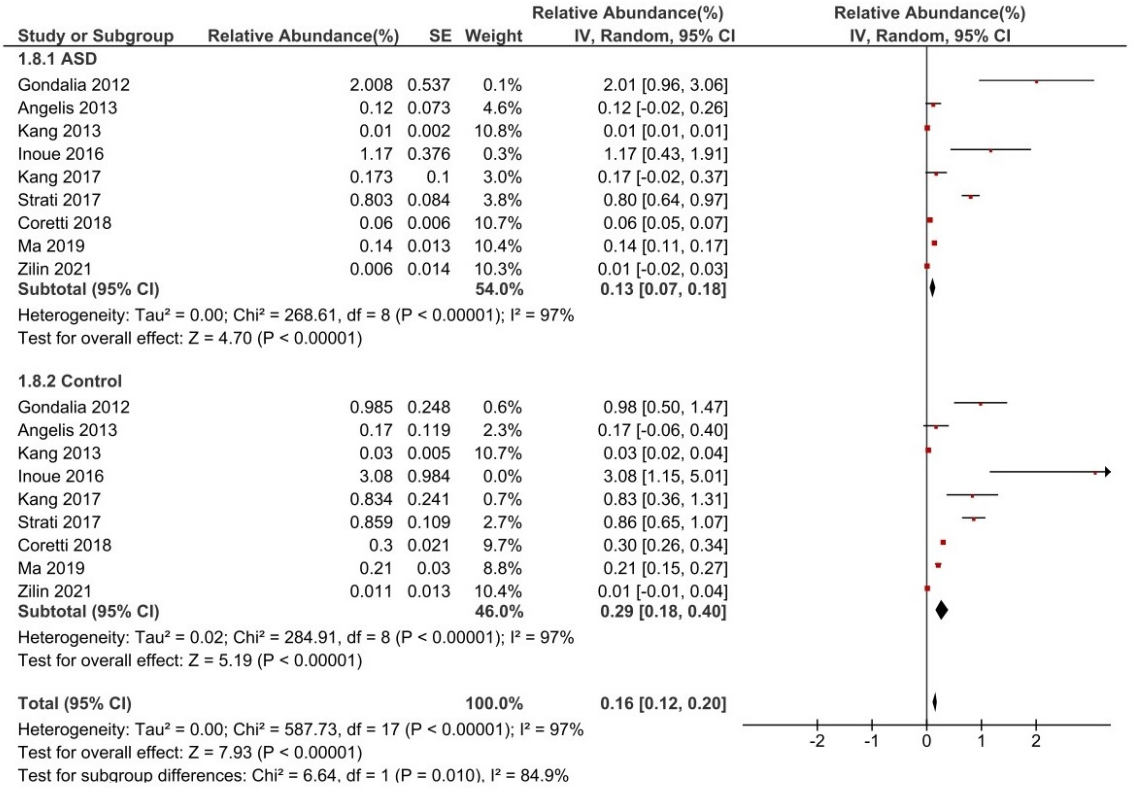

FIGURE 4.11: Forest plot for *Coprococcus* , depicting the lower relative abundance of this genus in ASD subjects as compared to the control group.

4.1.5.4 *Prevotella*

The random-effect model of meta-analysis included six studies for Prevotella. The output expressed the level to be 0.17% (95% CI: 0.02, 0.32) in ASD subjects and 0.34% (95% CI: 0.09, 0.60) in the control group.

Heterogeneity was found to be high between the included studies for ASD group (I^2=87%) and the control group (I^2=93%). By comparing the both groups, heterogeneity was found to be 23.8%.

The overall effect size was moderate (Z= 4.64) but significant (p>0.001). Both the groups showed a difference of 0.5 in bacterial percentage, expressing a lower level of Prevotella in ASD subjects as compared to the control group. Subgroup heterogeneity was not high, and results are not statistically significant. The forest plot is given in figure 4.12.

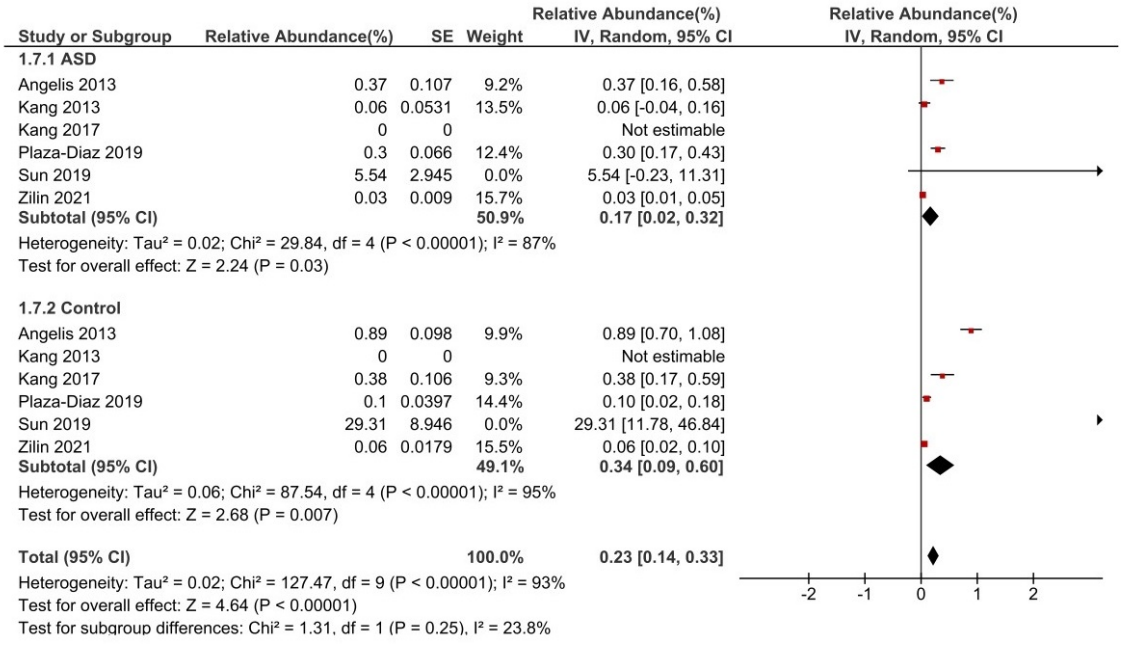

FIGURE 4.12: Forest plot for *Prevotella* , depicting the lower relative abundance of this genus in ASD subjects as compared to the control group.

The overall results obtained in terms of relative abundance in ASD and control group are shown in figure 4.13.

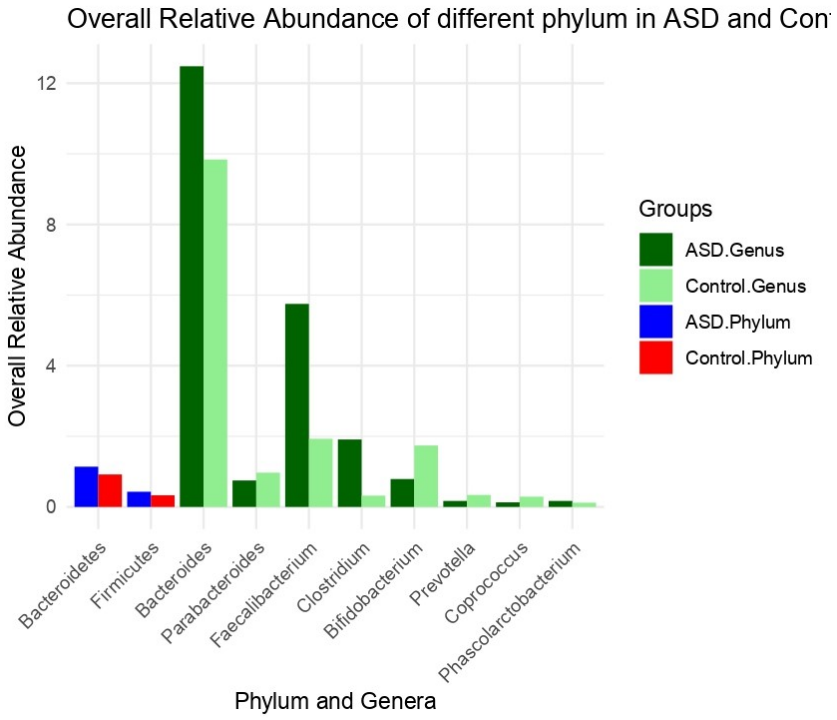

FIGURE 4.13: The graph depicting the relative abundance of different phylum and genus in ASD subjects as opposed to the control group.

4.2 Meta-genome Analysis (RQ 2)

The study aimed to investigate the gut microbial diversity in children with Autism Spectrum Disorder (ASD) compared to healthy controls, with a specific focus on understanding the potential differences in the composition and function of the gut microbiome between these two groups of ages 2 to 9 years. Various contributors play a significant role in the development of ASD symptoms, and we preferred children because variations in gut microbial composition are more evident in this age group. Studying early childhood microbial differences may provide insights into potential biomarkers and therapeutic targets for ASD management. To achieve this, we employed metagenome analysis using 16S rRNA sequencing, a powerful technique that allows for the identification and quantification of microbial communities in the gut. This approach provides a comprehensive snapshot of the microbial diversity present in the gut, enabling researchers to compare the types and abundance of microorganisms between different groups.

4.2.1 DNA Extraction, PCR Amplification and 16S rRNA Gene Sequencing

260/280 ratio of nucleic acid showed the quality whereas concentration is shown in ng/ul and is represented in Table 4.6.

TABLE 4.6: Summary of DNA quantification in 4 samples.

Sr. No.	**Sample ID**	**Nucleic Acid 260/280**	**Nucleic Acid Conc. in (ng/ul)**
1	AH	1.89	770
2	AI	1.83	780
3	NF	1.79	720
4	NU	1.81	773

AH refers to austistic sample 1, AI refers to austistic sample 2, NF refers to normal sample 1, and NU refers to normal sample 2.

4.2.2 Illumina MiSeq Sequencing

Illumina MiSeq Sequencing was used to generate high-throughput sequencing data in several studies. This platform offers high accuracy and throughput, making it suitable for large-scale microbiome analysis.

Its paired-end sequencing capability enhances taxonomic resolution and enables the identification of microbial species with greater precision. The generated sequencing data were then processed using various bioinformatics tools to identify and quantify the microbial communities.

The quality control and filtering steps were crucial in removing low-quality reads and ensuring the accuracy of the downstream analysis. Sequence alignment, taxonomic classification, and diversity analysis were performed using computational pipelines,

allowing for a comprehensive assessment of gut microbial composition and functional potential.

The statistics of raw data of each sample are in the table 4.7.

TABLE 4.7: Raw data statistics.

Sample ID	Index	Reads	Yields (Gbases	Q30 %
AI	TCGTAGTA+ TTAAGGAG	150,800	0.0754	90.19
NF	TCGTAGTA+ TCTGCATA	157,412	0.07871	90.03
NU	TCGTAGTA+ CTCCTTCC	161,987	0.08099	89.82
AH	TCGTAGTA+ CAATCCTC	148,931	0.07447	89.77

4.2.3 Microbiota Alterations

The study reveals the differences in gut microbial composition in ASD children and normal ones, both at the level of phylum and genus. A total of 8 phylum and 95 genera were shown to be differentially abundant in both groups. Specifically, the ASD group had higher abundances of Firmicutes and lower abundances of Bacteroidetes at the phylum level. At the genus level, the ASD group had higher abundances of Escherichia and lower abundances of Faecalibacterium. These findings suggest that the gut microbiota of ASD children is distinct from that of healthy children.

TABLE 4.8: Phylum level differences between ASD and NT children.

Sr. No.	Phylum	%age in ASD	%age in NT	P-value (T test)
1	Actinobacteriota	16.154	6.004	0.2735
2	Bacteroidota	73.033	105.948	0.1158

3	Campilobacterota	0	0.367	0.5
4	Cyanobacteria	0	1.395	0.5
5	Desulfobacterota	0.207	0	0.5
6	Firmicutes	99.645	145.848	0.1184
7	Proteobacteria	10.518	12.303	0.04969
8	Verrucomicrobiota	0.419	0.067	0.3991

TABLE 4.9: Genus level differences between ASD and NT children.

Sr. No.	**Genus**	**%age in ASD**	**%age in NT**	**P-value (T test)**
1	Clostridium	3.163	0.246	0.45059
2	Eubacterium	1.596	0.003	0.4988
3	Ruminococcus	22.765	5.529	0.34832
4	Agathobacter	12.163	9.592	0.07489
5	Akkermansia	0.419	0.067	0.39906
6	Alistipes	2.753	0	0.5
7	Alloprevotella	0	9.777	0.5
8	Allisonella	0	0.357	0.5
9	Anaerostipes	1.552	0.608	0.2623
10	Anaerostignum	0.03	0	0.5
11	Anaerotruncus	0.015	0	0.5
12	Anaerovibrio	0	1.845	0.5
13	Asteroleplasma	0	0.38	0.5
14	Bacteroides	68.854	6.056	0.44415
15	Bifidobacterium	16.102	5.841	0.27846
16	Blautia	7.548	3.588	0.21751
17	Butyricicoccus	0.709	0.935	0.08697
18	Bilophila	0.179	0	0.5
19	Collinsella	0	0.0466	0.5

20	Coprococcus	0.209	4.353	0.46946
21	Comamonas	0.178	0	0.5
22	Campylobacter	0	0.596	0.5
23	Catenibacterium	0	1.608	0.5
24	CAG-352	0.2041	0	0.5
25	Colidextribacter	0.344	0	0.5
26	Dialister	2.605	6.605	0.26084
27	Dorea	1.651	4.159	0.25943
28	Desulfovibrio	0.028	0	0.5
29	Erysipelato Clostridium	0.141	0.076	0.18528
30	Escherichia-Shigella	2.687	0.55	0.3715
31	Eisenbergiella	0.305	0	0.5
32	Erysipelotrichaceae_UCG-003	0.27	0	0.5
33	Enterococcus	0	0.162	0.5
34	Enterobacter	0.347	0.043	0.4215
35	Eggerthella	0.051	0	0.5
36	*Faecalibacterium*	4.966	10.658	0.2224
37	Flavonifractor	0.564	0.002	0.4977
38	Family_XIII_UCG-001	0	0.029	0.5
39	Family_XIII_AD3011_group	0	0.011	0.5
40	Fusicatenibacter	3.225	0.533	0.3957
41	Gastranaerophilales	0	1.29	0.5
42	Haemophilus	0.172	0.268	0.1368
43	Holdemanella	0	0.547	0.5
44	Holdemania	0.045	0	0.5
45	Hungatella	0.069	0	0.5
46	Howardella	0	0.035	0.5
47	Intestinimonas	0.089	0	0.5
48	Intestinibacter	0.073	0	0.5
49	Klebsiella	3.257	0.167	0.4674

50	Kosakonia	0.565	0	0.5
51	Kluyvera	0.059	0	0.5
52	Lachnospiraceae_ NK4A136_group	2.688	0.743	0.3283
53	Lachno*Clostridium*	2.27	0.488	0.3652
54	Lachnospiraceae_UCG-004	0.143	0.156	0.02766
55	Lachnospira	4.44	0.479	0.4316
56	Lachnospiraceae_UCG-008	0	0.207	0.5
57	Lachnospiraceae_ ND3007_group	0	0.157	0.5
58	Lachnospiraceae_ FCS020_group	0.037	0	0.5
59	Lachnospiraceae_ UCG-010	0	0.097	0.5
60	Lactobacillus	0.281	1.993	0.4108
61	Libanicoccus	0	0.063	0.5
62	Lactococcus	0.028	0	0.5
63	Muribaculaceae	0	0.508	0.5
64	Megamonas	1.598	0	0.5
65	Megasphaera	0.143	0	0.5
66	Marvinbryantia	0	0,057	0.5
67	Monoglobus	0.568	0	0.5
68	Oribacterium	0	0.786	0.5
69	Oscillibacter	0.782	0	0.5
70	Odoribacter	0.456	0	0.5
71	Prevotella	0	88.606	0.5
72	Parabacteroides	0.969	0.092	0.4397
73	Phascolarctobacterium	0.844	2.601	0.3002
74	Prevotellaceae_ NK3B31_group	0	0.501	0.5
75	Paracoccus	0	0.024	0.5

76	Proteus	0.023	0	0.5
77	Roseburia	12.173	2.92	0.3501
78	Rikenellaceae_ RC9_gut_group	0	0.538	0.5
79	Romboutsia	0	0.294	0.5
80	Ralstonia	0.021	0	0.5
81	Succinivibrio	0	10.366	0.5
82	Subdoligranulum	2.747	0.811	0.3172
83	Streptococcus	0	1.368	0.5
84	Sutterella	1.05	0.543	0.1962
85	Sellimonas	0.081	0	0.5
86	Senegalimassilia	0	0.037	0.5
87	Stenotrophomonas	0	0.034	0.5
88	Slackia	0	0.016	0.5
89	Tyzzerella	0.403	0	0.5
90	uncultured	1.063	3.847	0.3284
91	UCG-005	0.641	0	0.5
92	UCG-002	0	0.372	0.5
93	UCG-003	0.006	0.243	0.4843
94	UBA1819	0.043	0	0.5
95	Veillonella	0.06	0.236	0.3415

At the phylum level, *Proteobacteria* was significantly higher in normal children as compared to the autistic ones (Table 4.9, Figure 4.4). *Lachnospiraceae-UCG-OO4* was significantly higher in normal children than the ones with ASD (Table 4.9, figure 4.6) at the genus level. At the phylum level, *Proteobacteria* exhibited a statistically significant decrease in ASD children (10.518) compared to neurotypical (NT) children (12.303), with a p-value of 0.04969, indicating significance ($p < 0.05$). Similarly, at the genus level, *Lachnospiraceae*-UCG-004 was found to be lower in ASD children (0.143) compared to NT children (0.156), with a significant p-value of 0.02766. These

findings suggest notable microbial alterations in ASD children, potentially contributing to gut dysbiosis associated with the condition. Since *Proteobacteria* are known for their role in immune modulation and metabolic processes, their reduced levels in ASD children may indicate a dysregulated gut environment. Likewise, the decrease in *Lachnospiraceae*-UCG-004, a genus involved in short-chain fatty acid (SCFA) production, may have implications for gut-brain interactions in ASD. Further studies are needed to explore the functional consequences of these microbial shifts and their potential role in ASD pathophysiology.

TABLE 4.10: Gut microbial alterations in ASD and NT (neurotypical) children at phylum and genus level with significant differences (p value <0.05).

Variables	**ASD**	**NT**	**P value (T test)**
Phylum level			
Proteobacteria	10.518	12.303	0.04969
Genus level			
Lachnospiraceae-UCG-OO4	0.143	0.156	0.02766

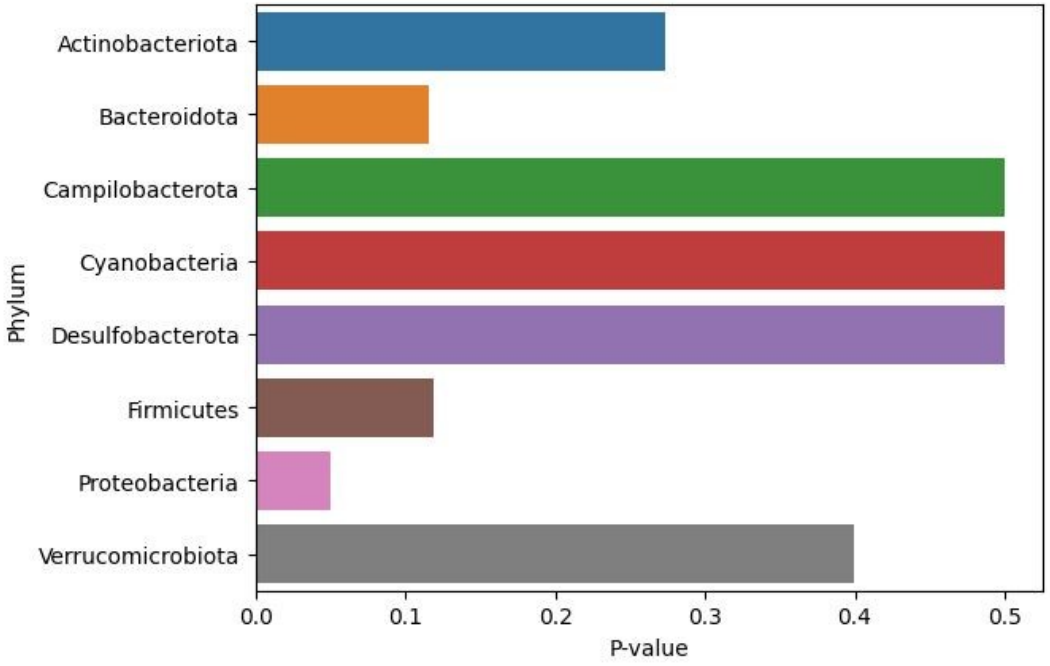

FIGURE 4.14: Bar plot of 8 phylum showing differential abundance in ASD and NT children. Proteobacteria shows significant decrease (p value 0.04) in ASD children as compared to the NT ones.

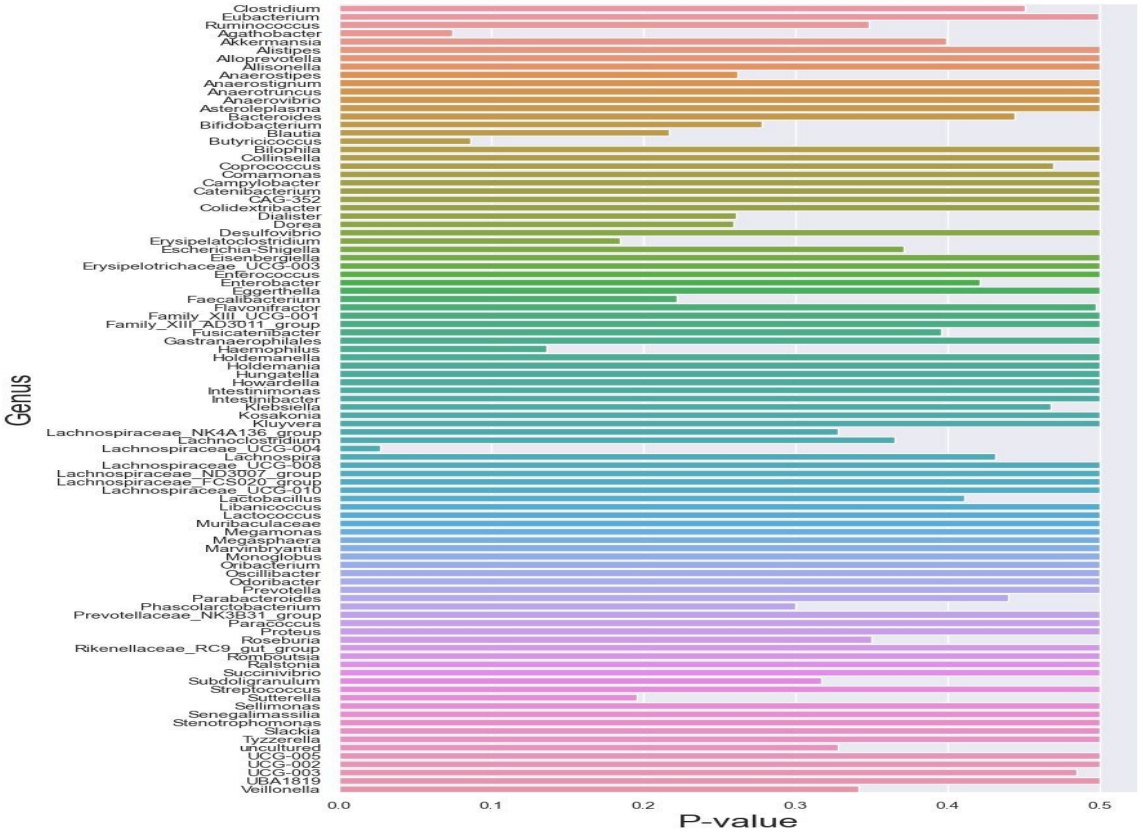

FIGURE 4.15: Bar plot of 95 genus showing differential abundance in ASD and NT children.

Lachnospiraceae-UCG-004 shows significant decrease (p value 0.02) in ASD children as compared to the NT ones. The gut microbial alpha diversity was compared between the two groups. Alpha diversity calculated through various indices is shown in table 4.10 and figure 4.6.

TABLE 4.11: Alpha diversity has been calculated through different indices. Shannon, Simpson and InvSimpson are shown to be highly significant.

Alpha diversity indexes	**AH**	**AI**	**mean**	**NF**	**NU**	**Mean**	**p-value**
Chao1	3776.193	3723.224	3749.708	5149.979	662 4.05	5887.015	0.13 89
se.chao1	3.580157	1.727755	2.653956	24.39499	47.0 935	35.74424	0.45 28

ACE	3776.293	3726.959	3751.626	5186.256	6604.344	589 5.3	0.13 92
se.ACE	30.44 108	29.99 825	30.21 967	35.23 002	39.24 066	37.23 534	0.06 597
Shannon	7.50 383	7.519 959	7.511 895	7.569 138	7.769 872	7.66 9505	0.00 6609
Simpson	0.998 952	0.999 052	0.99 9002	0.99 898	0.999 171	0.99 9076	2.36 E-05
InvSimpson	953.7 483	1055. 137	1004. 443	980.7 659	1205. 738	1093. 252	0.02 694
Fisher	775.5 838	748.8 164	762.2 001	1087. 041	1424. 098	1255. 569	0.15 27

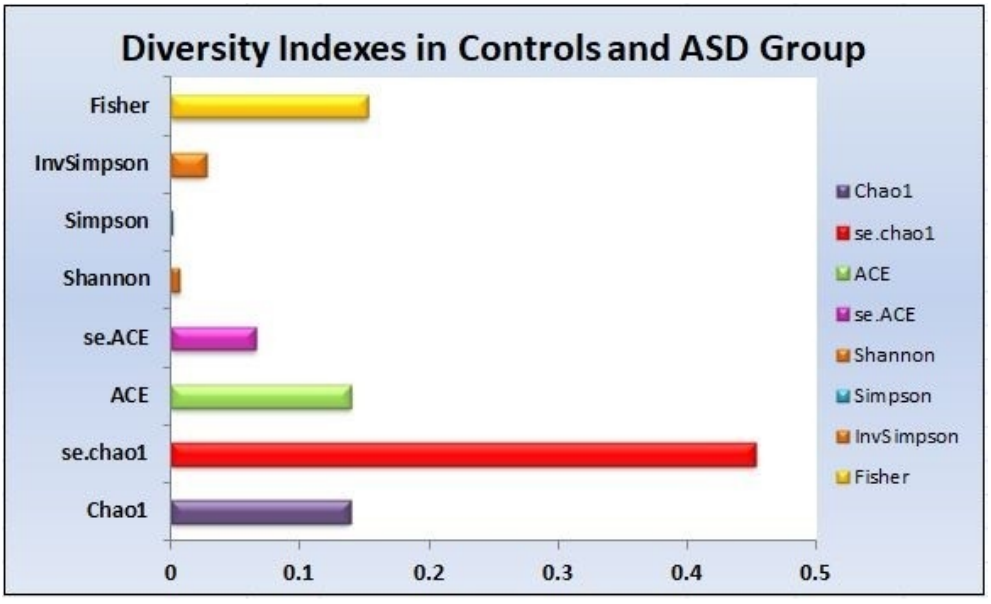

FIGURE 4.16: Bar plot of various alpha diversity indices in ASD and control group. Shannon, Simpson and InvSimpson are found to be highly significant.

A lower species richness was observed in ASD children compared to neurotypical (NT) children when assessing alpha diversity using indices such as Chao1, Shannon, Simpson, and Inverse Simpson. These metrics consistently indicated reduced microbial

diversity within the ASD group. This reduced biodiversity may suggest an imbalance in gut microbial communities, which could contribute to the altered metabolic or immune functions often associated with ASD.

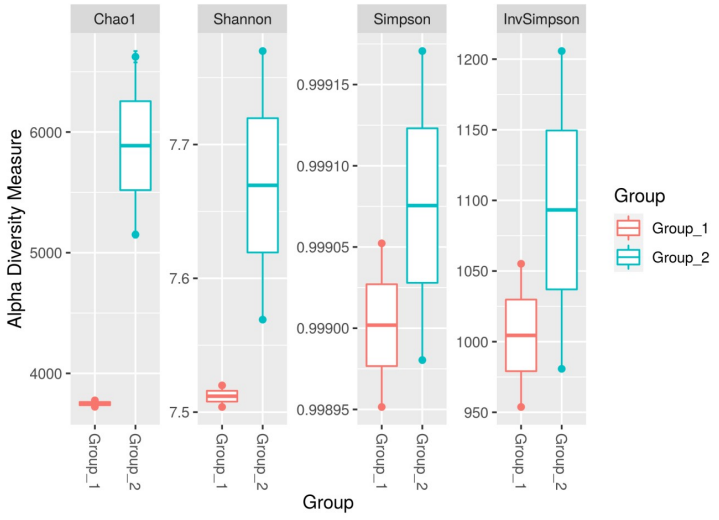

FIGURE 4.17: Alpha diversity assessed through Chao1, Shannon, Simpson, and InvSimspon. All alpha diversity measurement indexes exhibit lower values in ASD group as compared to the NT group depicting a lower specie richness in ASD group (Group 1 refers to ASD, and Group 2 refers to NT children)

The beta diversity calculated on weighted UniFrac distances and unweighted UniFrac distances showed that the ASD group was clustered apart from the NT group. The differential clustering or dissimilarity in clustering between the both groups exhibit that the both groups do not share the same gut microbial composition.

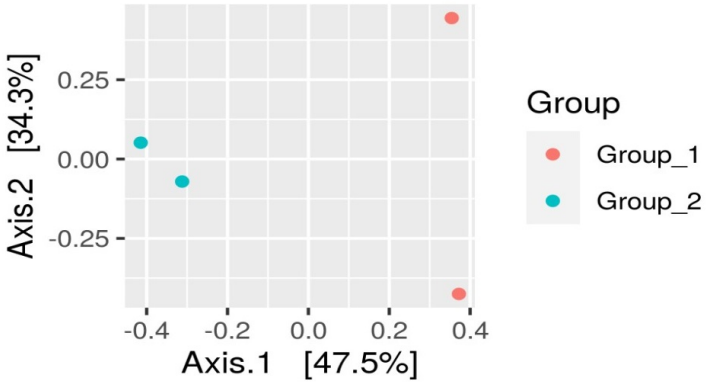

FIGURE 4.18: Unweighted unifrac distances exhibiting the differential clustering of ASD and NT group. ASD group is shown in red colour as group 1 and NT group in blue colour as group 2. ASD group is sparasely clustered from NT group.

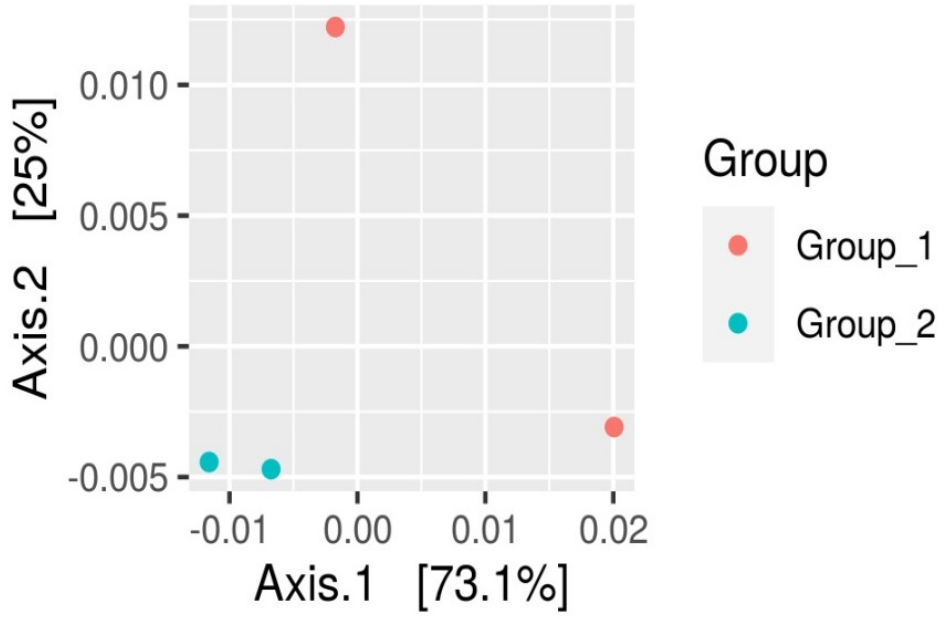

FIGURE 4.19: Weighted unifrac distances exhibiting the differential clustering of ASD and NT group. ASD group is shown in red colour as group 1 and NT group in blue colour as group 2. The dissimilarity in clustering of both groups show the differntial gut microbial composition in both groups.

The overall results obtained in the project has been summarized in figure 6.2.

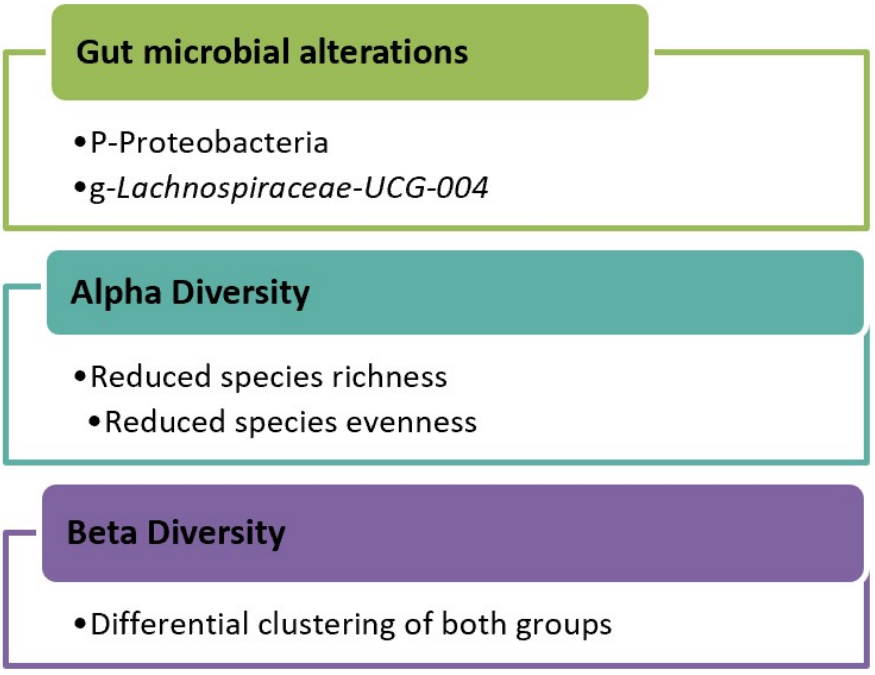

FIGURE 4.20: Figure depicts the overall result in terms of microbial alterations, alpha and beta diversity.

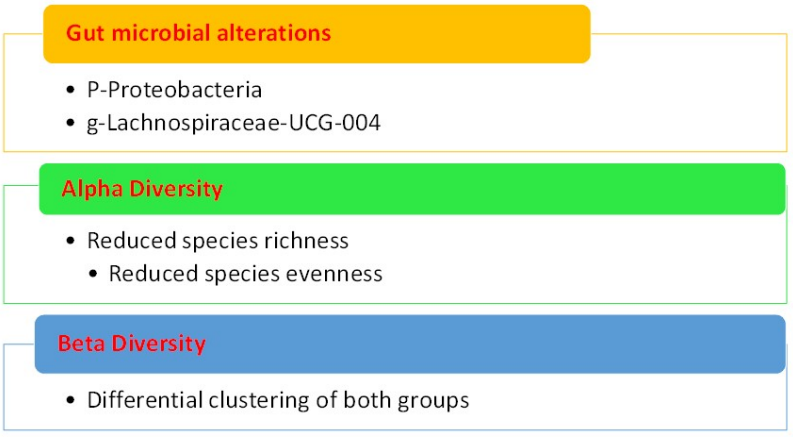

FIGURE 4.21: The conclusive results of metagenome analysis.

The Phylum Proteobacteria and genus Lachnospiraceae-UCG-004 has been found to be lower in ASD group than in NT group. Both species richness and evenness (alpha diversity) has found to be reduced in ASD group. Moreover both groups show difference in clustering in beta diversity depicting difference in microbial compostion in both groups. The conclusive results of metagenome analysis are depiected in figure below. After performing meta-analysis and metagenome analysis, certain bacterial genera were priorotized. The statistical results of meta-analysis depicted higher relative abundance of *Clostridium* and *Faecalibacterium*, and lower relative abundance of *Biofidobacterium*, and *Coprococcus*. On the other hand, the results of meta-genome analysis showed the differential abundance of Lachnospiraceae UCG-004 in ASD subjects in contrast to the control group.

4.3 Impact of Gut Microbial Metabolites (RQ 3)

4.3.1 Autism-Related Bacterial Species, Metabolites, and Their Reaction

The goal of our study is to identify the metabolites producing bacterial species and their potential role in autism spectrum disorder. We utilize the data gathered from

the literature review and generated from various databases. Compare these findings for processing and analysis.

The curated list generated through literature review and Human Metabolome Database (HMDB) consists of autism-related metabolites, information on how these metabolites levels vary in autistic persons than a healthy individual, and their potential function as shown in table 4.12.

TABLE 4.12: Curated list of Autism-related metabolites

S. No.	**Metabolites**	**Change in Autism**	**Potential Function**
1	p-cresol	Increased	Gut microbiota metabolite, possibly toxic
2	Glutathione	Decreased	Antioxidant
3	Cysteine	Decreased	Antioxidant precursor
4	Carnitine	Decreased	Fatty acid transport
5	Taurine	Decreased	Brain development and function
6	Hypotaurine	Decreased	Brain development and function
7	Melatonin	Decreased	Sleep-wake regulation
8	Lactate	Increased	Cellular energy production (may indicate dysfunction)
9	Branched-Chain Amino Acids (BCAAs)	Increased	Protein component
10	Valine	Increased	Part of BCAAs,

			involved in muscle growth and energy production
11	Leucine	Increased	Part of BCAAs, key in protein synthesis
12	Isoleucine	Increased	Part of BCAAs, important in hemoglobin production
13	Serotonin	Atypical levels	Mood, sleep, digestion
14	Hippurate	Changes observed	Gut microbiota metabolite
15	Kynurenic Acid	Increased	Neurotransmitter breakdown product
16	Quinolinic Acid	Increased	Neurotoxin
17	Folate (Vitamin B9)	Decreased	Vitamin B9
18	Vitamin B6	Decreased	Vitamin B6
19	Vitamin B12	Decreased	Vitamin B12
20	Short-Chain Fatty Acids (SCFAs)	Changes observed	Gut bacteria fermentation product
21	Acetate	Changes observed	Part of SCFAs, involved in energy production and gut health
22	Propionate	Changes observed	Part of SCFAs, influences glucose production
23	Butyrate	Changes	Part of SCFAs,

		observed	key in colon health and anti-inflammatory effects
24	Propiomazine	Unknown	Antipsychotic medication
25	3-hydroxy-3-(3-hydroxyphenyl) propanoic acid-O-sulphate	Increased	Metabolite involved in phenylalanine metabolism
26	HPHPA	Increased	m-tyrosine metabolite involved in phyenyl-alanine metabolism
27	Gliadorphin	Detected but not Quantified	Opioid peptide in gluten
28	Gluten exorphin B5	Abnormal leakage	Opioid peptide
29	Gluten exorphin C	Abnormal leakage	Opioid peptide
30	Gluten exorphin B4	Abnormal leakage	Opioid peptide
31	Gluten exorphin A5	Abnormal leakage	Opioid peptide
32	11Z-Eicosenoic acid	Increased in regressive autism	"Fatty acid potentially impacting brain function
33	2-Pentylthiophene	Detected but not Quantified	Volatile organic compound
34	Casomorphin	Increased	Dairy-derived opioid peptide
35	Ethyl propionate	Increased	Potential solvent or metabolic byproduct

36	4-Heptanone	Detected but not Quantified	Undetermined
37	Iminodiacetic acid	Reduced	Chelating agent, related to chemical exposure
38	3-Hexanone	Detected but not Quantified	Solvent
39	Dimethylethanol-amine	Treatment	Precursor to choline
40	2-Phenylethanol	Increased	Volatile organic compound
41	Toluene	Detected but not Quantified	Solvent exposure, potential neurotoxin
42	Adenylsuccinic acid	Deficiency	Intermediate in purine metabolism
43	Melatonin	Decreased	Sleep-wake regulation
44	Benzene	Detected but not Quantified	Toxic compound
45	Nonanal	Detected but not Quantified	Toxic compound
46	Oxalic acid	Increased	Metabolite that may affect kidney stone formation
47	2-Methylbutyryl-glycine	Increasaed	Related to amino acid and fatty acid metabolism
48	Indolylacryloyl-glycine	Increased	Tryptophan metabolite
49	Propionic acid	Detected but not Quantified	Short-chain fatty acid, affects gut

			microbiota
50	Acetylcysteine	Treatment	Antioxidant, used in medical treatments
51	Mercury	Undetermined	Heavy metal, neurotoxin

Initially targeted bacterial species are Clostridium, Bifidobacterium, Coprococcus, Faecalibacterium, and Lachnospiraceae UCG-004. Through VMH, Bacterial metabolites and reactions are isolated. But We found: 27 species of *Clostridium.* All 5 species of *Bifidobacterium.* All 3 species of *Coprococcus.* One species of *Faecalibacterium.* Unfortunately, *Lachnospiraceae* UCG-004 could not be identified due to its lack of a formal species name till date.

4.3.2 Isolation of Autism-related Bacterial Species

Using a Python script, data were extracted from multiple files to identify bacterial species specifically associated with autism. The script systematically filtered relevant microbial data, isolating key bacterial species, their associated metabolites, and biochemical reactions linked to autism.

Advanced bioinformatics tools and libraries, such as Pandas and Biopython, were employed to process large datasets efficiently. This approach facilitated the identification of microbial signatures potentially contributing to ASD pathophysiology. The list is shown in table 4.13.

TABLE 4.13: Bacterial species producing Autism related metabolites through VMH database

S. No.	**Name of Specie**	**Response**	**Metabolite Name**
1	*Clostridium bolteae*	Elevated	Acetone
2	*Clostridium perfringens*	Reduced	D-Lactic acid

3	*Clostridium aminobutyricum*	Elevated	vinylacetyl-CoA
4	*Bifidobacterium angulatum*	Reduced	10-Formyltetrahydrofolate

4.4 Pathway and Enrichment Analysis

The Overview of Enriched Metabolite Sets (Top 25) based on pathway enrichment analysis as shown in fig 4.22. The data suggests significant enrichment of certain metabolite sets in the analyzed dataset.

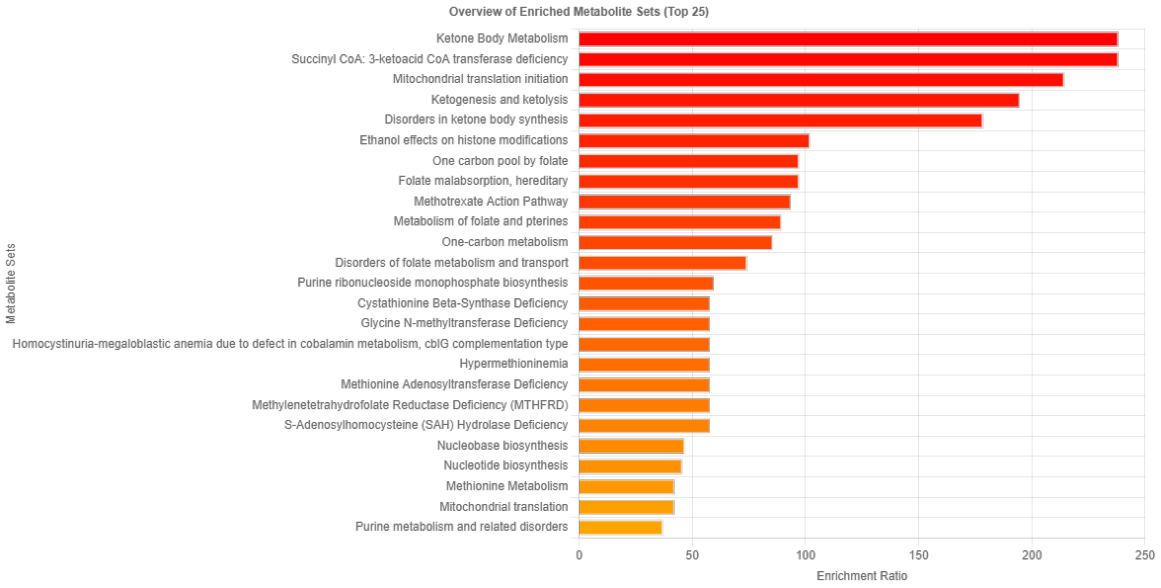

FIGURE 4.22: Overview of enriched metabolites sets (Top 25).

4.4.1 X-axis (Enrichment Ratio)

Represents the enrichment ratio, which indicates how much more frequently a metabolite set appears in the analyzed data compared to its expected frequency by chance. Higher ratios imply stronger enrichment. This metric helps in identifying significantly enriched metabolic pathways, providing insights into potential biological processes associated with ASD. A higher enrichment ratio suggests a stronger association between specific metabolites and autism-related microbial activity.

4.4.2 Y-axis (Metabolite Sets)

Lists the top 25 metabolite sets based on their enrichment scores, organized from most to least enriched. Includes pathways such as Ketone Body Metabolism, One-Carbon Pool by Folate, and Folate Metabolism, among others.

4.4.3 Color Gradient (Significance)

The gradient from red to orange highlights the level of statistical significance, with darker red representing more significant enrichment.

This analysis could provide insights into the metabolic underpinnings of autism, focusing on pathways involving:

- Acetone: Related to ketone body metabolism, possibly indicative of altered energy metabolism in autism.
- D-Lactic Acid: Associated with gut microbiota dysbiosis, frequently observed in autistic individuals.
- Vinylacetyl-CoA: Implicates mitochondrial or fatty acid metabolism.
- 10-Formyltetrahydrofolate: A key player in one-carbon metabolism and folate-dependent reactions, essential for neural development.

4.4.4 Ketone Body Metabolism

Acetone is a product of ketone body metabolism, a top-enriched pathway in the figure. Dysregulation of ketone body metabolism is linked to energy imbalance in the brain, which has been associated with neurodevelopmental disorders, including ASD. Recent studies have highlighted the potential role of altered ketone pathways in modulating neural activity and cognitive function in children with ASD. Furthermore, interventions targeting ketone body regulation are being explored as therapeutic strategies.

4.4.5 D-Lactic Acid & Metabolism of Folate

D-Lactic acid is a microbial metabolite produced during carbohydrate fermentation by gut bacteria. Elevated levels of D-lactic acid can lead to D-lactic acidosis, which may contribute to neurological and cognitive symptoms often observed in autism. The chart highlights enriched pathways related to folate metabolism (e.g., "One carbon pool by folate" and "Methotrexate Action Pathway"), where 10-Formyltetrahydrofolate plays a critical role. Folate is essential for brain development, methylation, and DNA repair. Impairment in folate pathways is linked to ASD and related symptoms.

4.4.6 Vinylacetyl-CoA & Mitochondrial Dysfunction

Vinylacetyl-CoA is involved in mitochondrial and energy metabolism. Mitochondrial dysfunction is frequently reported in ASD and can impair energy production in neurons, exacerbating neurodevelopmental issues.

Chapter 5

Discussion

ASD being a multifactorial disorder is presumed to be caused by both genetic and environmental factors [4]. Gut microbiota, affecting human physiology in numerous ways, is involved in the etiology of ASD, majorly by exhibiting the variation of certain bacteria in ASD children as compared to their neurotypically growing children [7]. Despite of the various studies conducted to evaluate the differences in the alterations of GM in ASD subjects and the healthy control, the results obtained are in-consistent. In order to conclude statistically significant results regarding the specific bacteria which show higher or lower abundance in ASD subjects as opposed to healthy controls, three meta-analyses have already been conducted [36, 37, 37].

In the present meta-analysis, various indicators such as uniform data collection, rigorous inclusion criteria, and robust statistical tools have been ensured in order to have errorless and precise results. Moreover, previously reported meta-analyses have been updated through this study, both in terms of number of included studies, and a better and in depth understanding of the variations in GM in children with and without ASD.

The findings of our meta-analysis revealed the higher RA of Bacteroidetes and Fermicutes in children with ASD as compared to the children without ASD. At the genus level, RA of Bacteroides, *Clostridium*, *Faecalibacterium*, and *Phascolactobacterium* is concluded to be higher in children with ASD as compared to children without ASD.

On the other hand, RA of *Bifidobacterium*, *Coprococcus*, Parabacteroides, and Prevotella is found to be lower in children with ASD as opposed to healthy controls. Out of these reported results, significant results have been found of higher RA of *Clostridium* and *Faecalibacterium* and lower RA of *Bifidobacterium* and *Coprococcus*.

Our results are in line with the previous studies, emphasizing the dysbiosis of certain GM in children with ASD. The higher levels of *Clostridium* has already been reported in various studies [78, 80, 83, 85, 87, 88, 90, 93, 94, 97, 101, 103, 104, 118]. Various studies conducted to assess the variations in gut microbial composition in ASD children as opposed to healthy controls have shown the higher RA of *Faecalibacterium* as reported by this current study [83, 85, 87, 88, 90, 93, 94, 101, 104, 113, 114, 117, 151].

The lower RA of *Bifidobacterium* as reported in this study is in consistency to the previous studies [81, 83, 85, 87, 88, 90, 93, 94, 101, 104, 113, 114, 121, 151]. The present study is in line with some previously conducted studies with respect to the lower RA of *Coprococcus* [85, 87, 88, 90, 94, 98, 101, 117, 151]. The dysbiosis in gut-microbiota-brain axis compromises the bi-directional communication between the gut and the brain. The usual modes of communication including hormonal pathways, and neurotrasnmitter pathways are badly affected, but the baseline interactions between these pathways and their causative role in the etiology of ASD is still obscure.

The results of the present study validates the dysbiotic environment in gut of the children with ASD as higher RA of harmful bacteria and lower RA of beneficial bacteria have been reported. *Clostridium*, a gram positive spore-forming bacteria, is reported to be in higher percentage in ASD children as opposed to the healthy control.

This genus is capable of synthesizing certain pro-inflammatory enterotoxins and neurotoxins, which have the potential to cross the blood-brain barrier and lead to serious pathological conditions related to ASD [39]. *Clostridium* leads to the increased production of p-cresol which in turn leads to raised levels of propionate, thus causing peripheral inflammation and ASD like behavior [40]. *Clostridium* produces toxin b, which confers pathological structural variations including increased number of dendritic spines along with the cell death of certain neurons [41].

Faecalibacterium, a gram negative bacteria, harbors a commensally beneficial relationship within the GIT, and is associated with systemic immune dysfunction. This genus regulates the expression of certain genes related to interferon (IFN) gamma, a cytokine, which has roles in synapse formation and brain plasticity. Early exposure to this cytokine as well as the increased concentrations have been reported to badly affect neuronal functions, disrupting the immune system balance, and results in ASD-like behaviors [42].

Bifidobacterium is a gram positive bacteria and is protective in nature for GIT. This genus is involved in the production of indole-3-lactic acid, which hinders the growth of certain harmful pathogens like Eschericia coli, thus reducing inflammation and protecting the GIT environment. The positive impact of *Bifidobacterium* is further strengthened by the fact the administration of *Bifidobacterium* rich probiotics ameliorates the autistic behavior [43].

Moreover, *Bifidobacterium* produces Gamma-Amino butyric acid (GABA), which plays a critical role in glutamate (excitatory neurotransmitter) metabolism [44]. The lower abundance of this genus thus leads to lower concentrations of GABA, and glutamate, therefore aggravating anxiety, social disruptions, and autism related neurotypical profile. Thus, the lower RA of this genus disturbs the healthy gut environment and contributes to the etiology of ASD [45].

Coprococcus is a gram positive fermenting bacteria which produces butyrate. Butyrate inhibits nuclear factor kappa B (NFKB), thus preventing inflammation [46]. The protective nature of intestinal mucosa, and intestinal motility is ensured by butyrate as it reduces the production of reactive oxygen species, inhibits excessive cell proliferation and differentiation. In this perspective, reduced levels of *Coprococcus* leads to lower butyrate levels, thus causing the dysbiotic environment and compromising the healthy GIT [42].

ASD has emerged as a multifactorial disorder and various risk factors including genetic, epigenetic, and environmental have been speculated in the etiology of this complex spectrum disorder. This study has been designed in order to evaluate the differ-

ences between autistic children and healthy ones, mainly by comparison of their gut microbiota, as ample evidences support the putative role of gut microbiota in this multifactorial disorder even though the exact mechanism of involvement remains obscure [6].

16S rRNA sequencing of 2 autistic children and 2 nneurotypically growing children was assessed in the present study to delineate the differences in gut microbial composition in Pakistani children. The present study shows that the autistic children harbor a less diverse gut microbiota along with the reduced richness, as compared to the control group. A decrease in alpha and beta diversity in autistic children has been found as compared to the control group.

The present study also reports the significant deviations and alterations in the gut microbial composition in autistic children as compared to the neurotypically growing ones. At the phylum level, relative abundance of proteobacteria was lower in ASD subjects and at genus level, the abundance of Lachnospiraceae UCG 004 was found to be significantly different between autistic children and neurotypically growing ones. The genus was found to be in lower abundance in autistic subjects as compared to the normally developing ones.

The microbes reside in the human gut, and are associated with human health and disease conditions. A layer of mucus separates the gut microbes from the epithelial layer. In ideal conditions, the gut microbes reside in the outer layer of mucus and take essential nutrients from it for their survival, and do not cross it to enter in to epithelial cells [157].

Gut microbes can interact to the host via various pathways, including metabolic products. These metabolic products are produced due to fermentation, and degradation of dietary fiber, and include Short Chain Fatty Acids (SCFAs) like acetate, butyrate, and propionate. In addition to it, certain essential vitamins are also produced by human gut microbes [158].

Phylum Proteobacteria are the gram-negative bacteria that possess lipopolysaccharide in their outer membrane. A total of six classes have been defined of the phylum based

on the 16S rRNA phylogenetic analysis. The phylum is renowned due to certain important human pathogenic genera like Rickettsia, Neisseria, Salmonella, Shigella, and Escherichia. Members of Proteobacteria are found to be increased in disease condition, and associate with inflammatory condition of host [159]. The lower RA of Proteobacteria has been obtained in this study which is in consistent to a previously reported study [96].

Lachnospiraceae family belongs to the cluster XIVa of phylum Firmicutes, and all members are anaerobic fermenters [160]. Lachnospiraceae UCG-004 produces butyrate, which has the potential to activate the G protein-coupled receptor (GPR) 43, which in turn modulates the inflammation. It can stimulate glucagon-like peptides (GLP) 1 and gastric inhibitory polypeptide, increase the insulin sensitivity and metabolism of glucose, and regulate appetite [161]. GPR 43 binding protects the liver by suppressing colon inflammation and insulin signal transduction is down-regulated in adipose tissue [162].

Butyrate up-regulates the tight junctions, and activates the enterocytes for mucin production. This has an overall effect of maintaining and strengthening the intestinal barrier [163]. It induces regulator T cells, and down-regulates certain important pro-inflammatory cytokines, and Toll Like Receptors 4 (TLR4), thereby exerting an anti-inflammatory response [164].

Butyrate effectively regulates glucose and energy homeostasis by stimulating gluconeogenesis [158]. Butyrate is bestowed with the ability to prevent the gut microbiota dysbiosis because oxygen balance is generated in the gut as butyrate with the virtue of β oxidation lets epithelial cells to use huge amount of oxygen [165].

The lower abundance of Lachnospiraceae UCG 004 presumably leads to lower butyrate levels, which is a key metabolite in maintaining the integrity of microbiome-gut-brain axis [166]. The expression of tight junction-associated proteins is upregulated by Adenosine Monophosphate-activated (AMP) protein kinase which in turn is regulated by butyrate [167]. Strengthening of mucosal immunity and restoration of BBB premeability is also owed to butyrate because of its ability to upregulate the histone acetylation and expression of tight junction proteins [168].

Butyrate has also the ability to alter the expression of tyrosine hydroxylase gene, thus indirectly regulating the sysnthesis of dopamine [101]. Taken together, butyrate can regulate the gut-brain axis and the lower levels can lead to disastrous spectrum in ASD. Moreover, the proliferation of pathogenic gut microbiota and lower abundance of healthy gut microbiota leads to dysbiosis in autistic children. This dysbiosis is associated with the pathogenic condition of GI disorders.

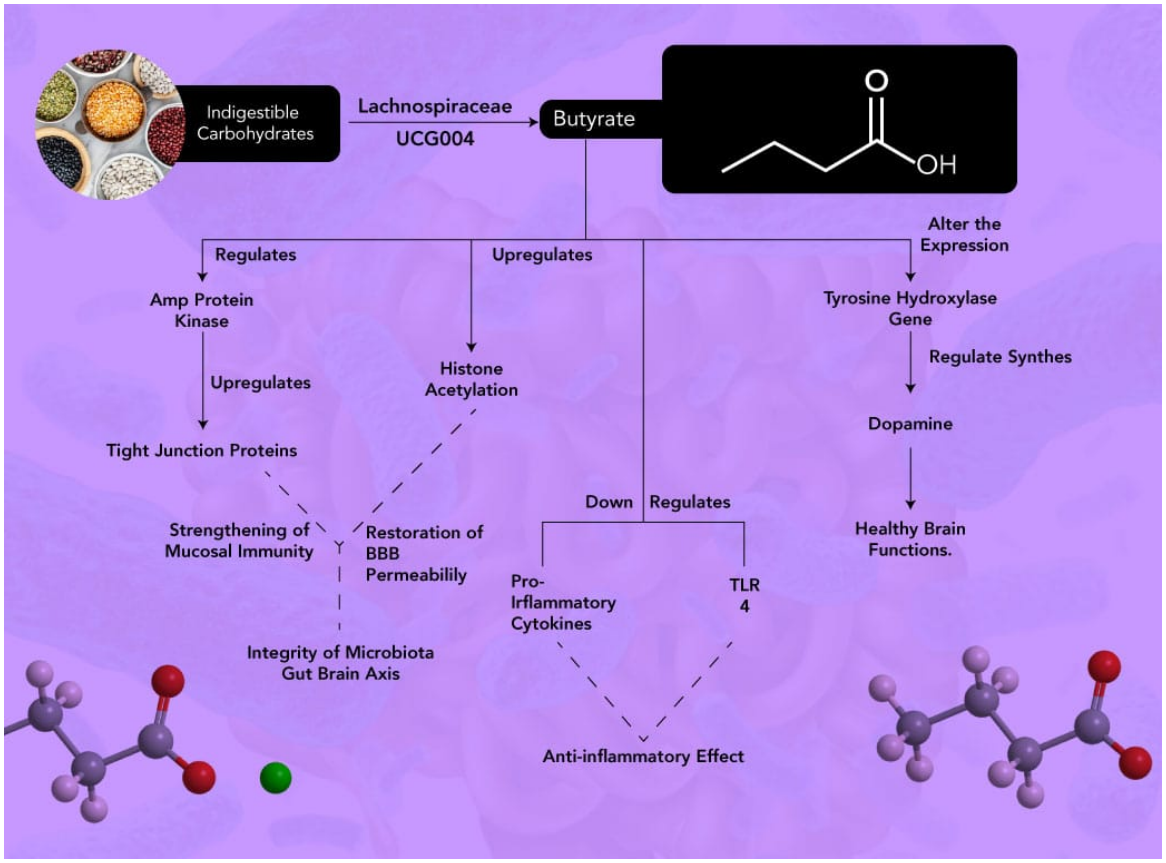

FIGURE 5.1: Role of *Lachnospiracece ucg004* in human body

Thus it is speculated that the gut inflammation and GIS could be reversed in ASD subjects by maintaining and restoring the healthy levels of Lachnospiraceae UCG 004. Certain previous studies also report the lower abundance of members of Lachnospiraceae in autistic subjects [83, 101, 102, 115, 118].

By considering already reported studies and findings of the present research, it could be concluded that the members of Lachnospiraceae significantly contribute in maintaining a healthy gut environment and their dysbiosis is directly correlated with GI disorders in ASD children.

The number of samples evaluated in the present study are less than the usual number of samples reported in meta genome analysis in ASD studies. The main reason behind this has been the taboo associated with this disorder in Pakistan and subsequent unwillingness of the parents to provide fecal samples. This unwillingness basically owes to the social, emotional, and cultural aspects in Pakistan, where patients with such disorders are still stigmatized and thus there is a usual denial from acceptance of such disorders. However, the results of this preliminary study are statistically significant and correlated [1, 168].

Gut microbes produce certain metabolites which play pivotal role in human brain physiology as they are bestowed with the ability to cross the BBB. These metabolites could be one of the probable mechanisms through which gut microbes could affect the brain functioning and play their role in onset or pathophysiology of ASD. Out of the five identified genera, eighteen metabolites have been filtered out by various species. However, further analysis could not establish their exact linkage with ASD.

Metabolic Role of Metabolites in Autism

To explore the role of autism-related metabolites, we have to compare the KEGG pathway with the literature. The metabolic role of metabolites in autism is as follows:

Acetone

Acetone (Fig. 5.2) belongs to ketone produced during the breakdown of fatty acids in the liver, particularly carbohydrate level falls. It is one of the byproducts of ketogenesis, in which the liver generates ketone compounds to use as an alternative energy source [152].

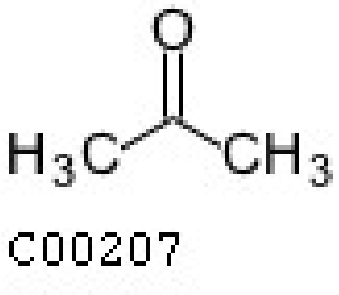

FIGURE 5.2: Structural Formula of Acetone.

In one study, altered microbial fermentation as shown in Fig. 5.3, including the production of compounds such as acetone, has been observed in individuals with autism spectrum disorder (ASD). The gut-brain axis plays a role in ASD, where microbial metabolites can affect neurological function and development.

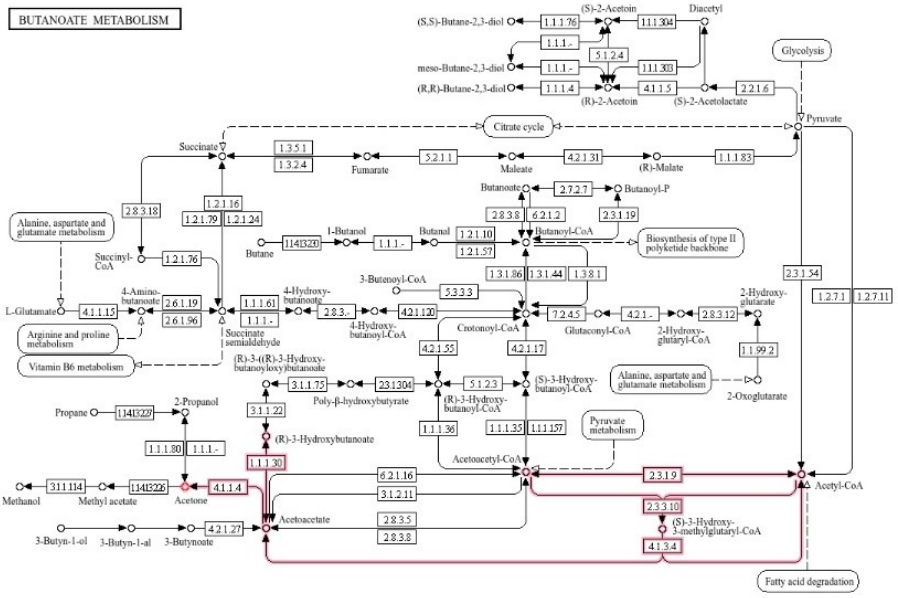

FIGURE 5.3: Butanol metabolism (highlighted part show ketone body biosynthesis that play crucial role in autism)

An imbalance in gut microbiota and their metabolic byproducts, such as acetone, may contribute to behavioral and cognitive symptoms associated with autism. Clostridium bolteae, a species noted for its increased presence in the gut microbiota of some individuals with ASD, has been studied for its potential contribution to the altered metabolic profile observed in autism. Acetone production by this species could influence the gut environment and impact neurodevelopment via the gut-brain axis. It also impacts the synthesis and regulation of neurotransmitters. Further disruptions in fatty acid metabolism and ketone body production can lead to energy deficits in the brain, which may contribute to the neurodevelopmental challenges observed in ASD [153].

D- lactic Acid

D-lactic acid is one of two stereoisomers of lactic acid, produced by specific gut bacteria during carbohydrate fermentation. Clostridium and Bifidobacterium species are

among the key contributors to D-lactate production. Under normal physiological conditions, D-lactate remains at low concentrations due to efficient metabolic regulation. However, in cases of gut dysbiosis, excessive bacterial fermentation can lead to D-lactate accumulation, potentially contributing to metabolic disturbances. Elevated D-lactate levels have been implicated in neurological symptoms and systemic inflammation, highlighting its potential role in conditions such as ASD and other gut-brain axis disorders.

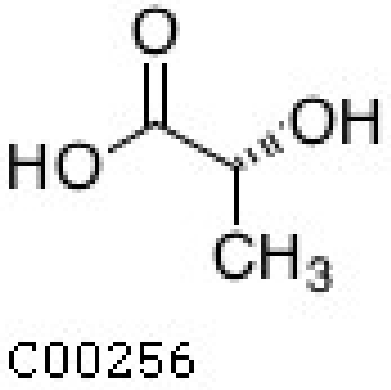

FIGURE 5.4: Structural formula of D-Lactic acid

Lactic acid is produced during glycolysis as shown in fig. 5.5, where glucose is converted to pyruvate, and under hypoxic conditions, pyruvate is reduced to lactic acid by lactate dehydrogenase (LDH).

This process is crucial for energy production, especially in tissues with high energy demands, such as the brain. In the brain, lactic acid is primarily produced by astrocytes and serves as an energy substrate for neurons. It is transported from astrocytes to neurons via monocarboxylic acid transporters (MCT).

This transport is essential for maintaining neuronal function and energy metabolism. Lactic acid can activate GPR81, a receptor that promotes anti-inflammatory effects and inhibits GABA neurotransmission. This modulation can affect ASD symptom such as sleep, learning, and memory, which are critical for cognitive functions. Lactic acid also plays a role in the microbiota-gut-brain axis, where it can influence brain function through gut microbiota.

The colonization of lactic acid-producing bacteria has been shown to exert antidepressant effects, indicating a potential pathway for regulating mood disorders. Lactic acid enhances calcium currents in neurons by binding to NMDA receptors, which activate intracellular signaling pathways. This process upregulates genes related to neuroplasticity, such as brain-derived neurotrophic factor (BDNF), which is vital for learning and memory [154].

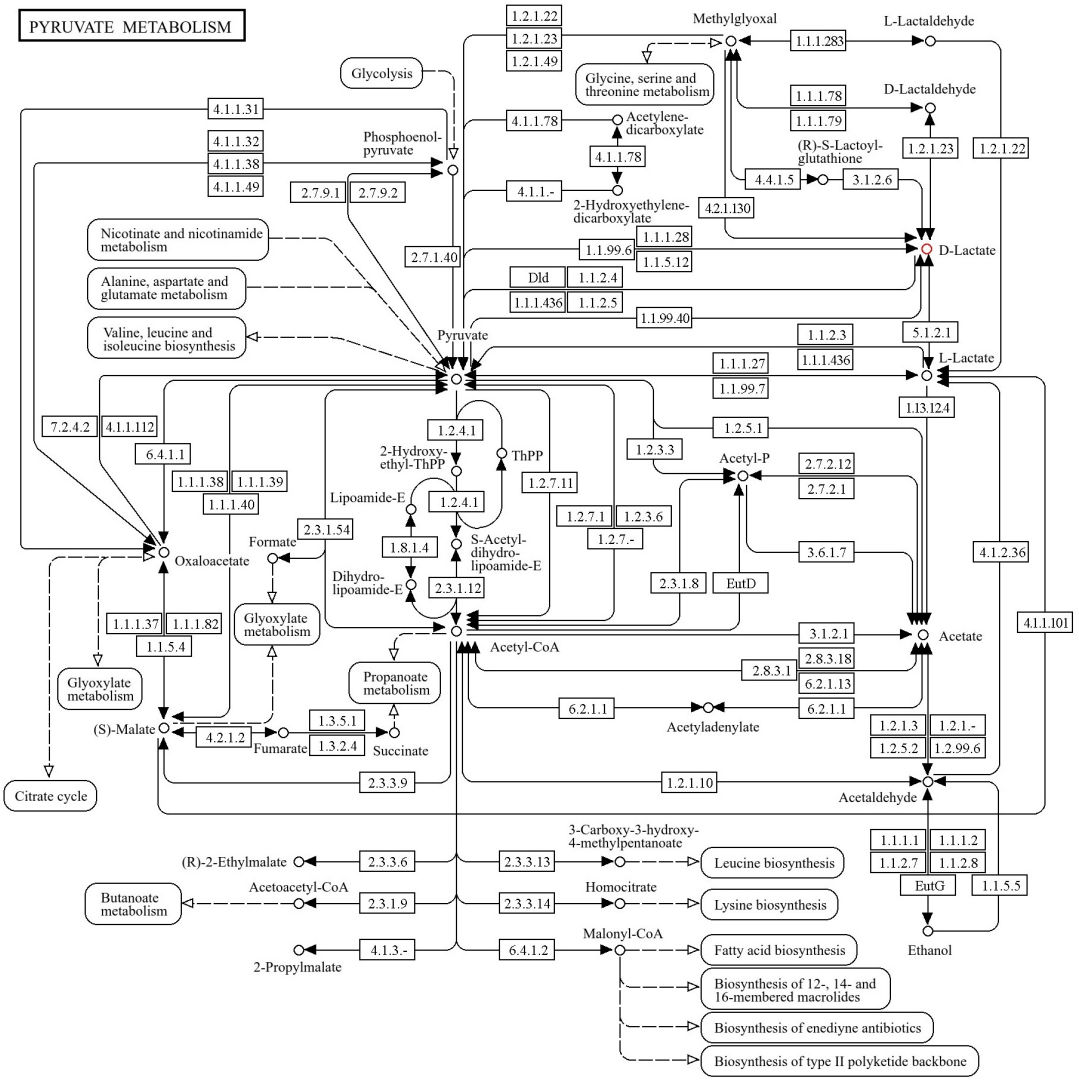

FIGURE 5.5: Pyruvate metabolism

Vinylacetyl-CoA

Cholesterol and isoprenoid metabolism have been implicated in the development of autism spectrum disorders (ASDs) through various mechanisms. Vinylacetyl-CoA

(Fig. 5.6) is a precursor it. for the synthesis of fatty acids, and abnormalities in its metabolism could lead to altered levels of fatty acids in the brain [155].

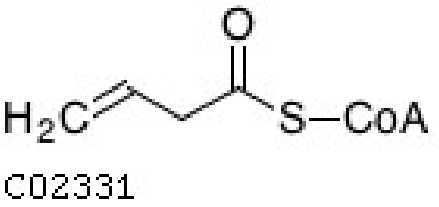

FIGURE 5.6: Structural formula of Vinylacetyl-CoA

Its reduced production leads to reduced synthesis of fatty acids, and abnormalities in its metabolism could lead to altered levels of fatty acids in the brain and also reduced production of amino acids as shown in Fig. 5.7. These imbalances also contribute to neuroinflammation, a process associated with autism [155].

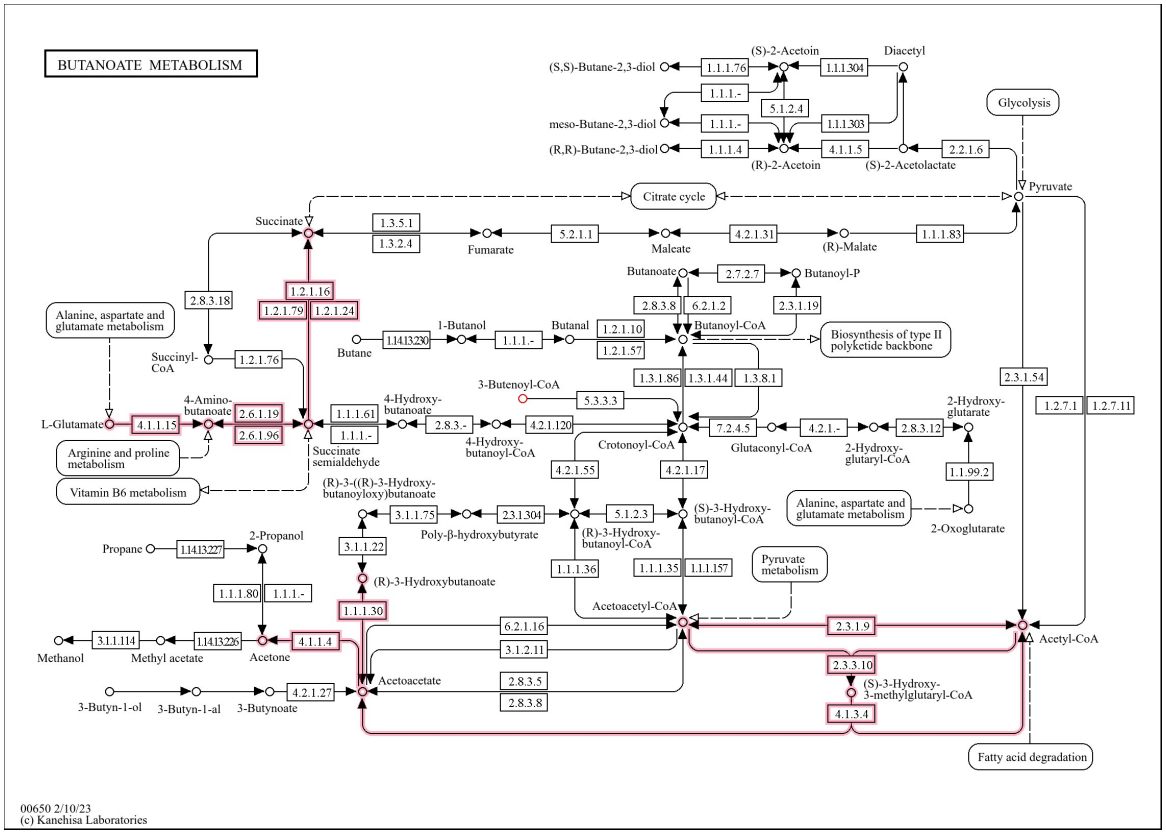

FIGURE 5.7: Fatty acid and amino acid synthesis reaction

10-Formyltetrahydrofolate

10-Formyltetrahydrofolate (Fig. 4.16) is a critical molecule in one-carbon metabolism, where it acts as a donor of formyl groups during the synthesis of purines, essential for DNA and RNA formation. Additionally, it is involved in the methylation cycle, which is vital for regulating gene expression, especially during early neural development. The proper functioning of these processes is crucial for cognitive and behavioral functions, which are often impaired in individuals with ASD [156].

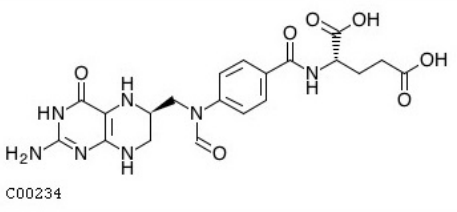

FIGURE 5.8: Structural formula of 10-Formyltetrahydrofolate

In the Folate Metabolism network, the key role player is 10-Formyl-Tetrahydrofolate (THF) in one-carbon metabolism. In this pathway, 10-Formyl-THF is an essential intermediate for purine biosynthesis to help in the transfer of formyl groups for nucleotide formation. It also helps in the formation of 5,10-Methylene-THF and 5,10-Methenyl-THF as shown in Fig. 4.17, which play vital roles in DNA synthesis and repair mechanism [153].

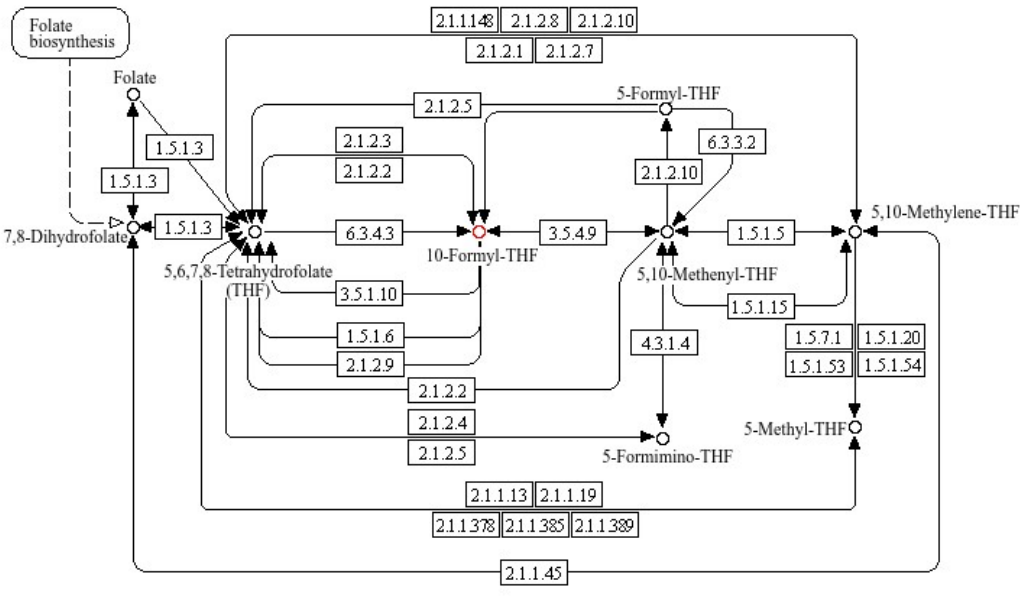

FIGURE 5.9: One carbon pool by Folate

This pathway is integral to DNA synthesis, methylation, and neurotransmitter production, all of which are crucial for brain development. Reduced 10-Formyltetrahydrofolate levels could lead to insufficient methylation, altering gene expression patterns that are critical for normal brain development. Impairments in this pathway can lead to developmental delays and ultimately to Autism.

The study encountered several limitations that should be acknowledged. One significant challenge was the limited sample size of children with Autism Spectrum Disorder (ASD) and neurotypical children. Recruiting participants proved difficult due to accessibility issues and the availability of eligible individuals, which constrained the breadth of the analysis. Additionally, financial, domestic, and societal barriers posed significant challenges. Limited funding restricted the scope of the study, while societal unawareness regarding ASD and its symptoms made community engagement and participant recruitment particularly challenging. These constraints affected the study's logistics and overall execution. However, it must be considered that the primary goal of the objective was to work on taxonomy assignment rather than functional annotations.

Another notable limitation was the inability to conduct in-vivo validation. While the study employed advanced meta-analysis and metagenome sequencing techniques to investigate the microbial composition and diversity, confirming the functional roles of identified metabolites and their direct implications in ASD was not feasible. Furthermore, due to technical and resource constraints, metabolome analysis could not be performed. This omission hindered the study's ability to provide a comprehensive examination of the biochemical interactions between gut microbiota and host metabolism.

The findings provided a general overview of metabolic pathways potentially associated with ASD. However, these insights did not allow for the precise identification of disrupted pathways or their mechanistic linkages to the condition. This limitation underscores the need for future research to integrate metabolome analysis, in-vivo validation, and larger sample sizes to enhance the understanding of gut microbial composition and its role in ASD pathogenesis.

Chapter 6

Conclusion and Future Work

6.1 Conclusion

Autism Spectrum Disorder (ASD) constitute a complex set of neurodevelopmental disorders manifested by poor communication skills, social withdrawal, and repetitive or restrictive behavior, interest or activity. The prevalence of ASD shows a sharp increase in the recent past, and it has been reported by Centre for Disease Control (CDC) that in the US 1 out of every 36 children is affected with ASD.

Currently, the most reliable tool to diagnose ASD patients is Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM V), in which the ASD patients are identified and characterized based on their abilities and difficulties in developing verbal and non-verbal communications, and social interactions. A wide range of symptoms is exhibited by ASD individuals, making a complex phenotype of the disorder.

The early diagnosis of ASD is highly desirable so that the symptoms could be treated well in time, and available treatment approaches could be offered, however the lack of clinical or molecular biomarker, the diversification in symptoms, and accurate diagnostic tools hinder the in time diagnosis. There is a range of phenotypes that is associated with autism, and outcome of the symptoms also vary between different individuals thus making it a spectrum disorder.

Moreover, certain co-morbid conditions are also linked to ASD, contributing to the heterogeneous and complex phenotypes. The financial burden associated with ASD is huge, expected to be $450 billion in 2025 in US. ASD not only suffers the finances of the individuals, but also the family and societal life. The lack of acceptance of ASD individuals from the community, the linked comorbid conditions along with the core ASD symptoms, and financial and social pressures deeply affect the overall life standards, physical, and mental health of caretakers along with the ASD patients. In addition to it, there is no reliable treatment protocols for ASD individuals.

Initially, autism was identified as a psychiatric or behavioral disorder that primarily arose due to lack of parental attention. With the advent in scientific research, it was known that ASD arises due to certain biological reasons, thus it emerged out as a neurological disorder as opposed to the earlier concept of behavioral or emotional causes. Various risk factors such as genetic, epigenetic, and environmental are linked to underpin the pathophysiology of ASD.

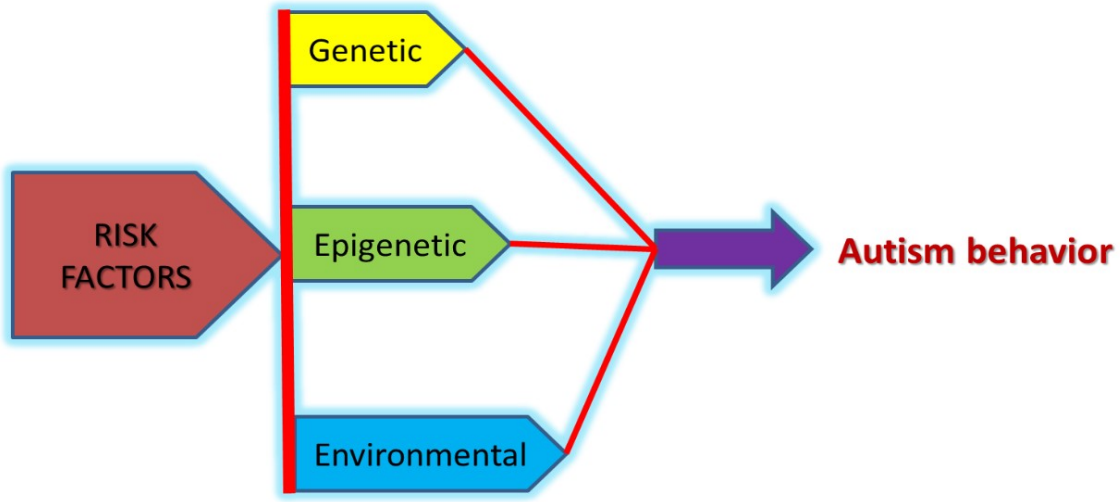

FIGURE 6.1: Various risk factors in the onset and complexity of ASD.

More recently, environmental factors including prenatal viral infection, maternal diabetes, certain toxins, heavy metals, environmental insecticides and pesticides, immunological proteins, food contaminants, parental age, maternal smoking and alcohol consumption, and gut microbiota have emerged as the potential risk factors for ASD.

The genetically susceptible patterns may become target of environmental threat and result in dysregulations of neurodevelopmental pathways, but these complex interactions are difficult to be identified due to a high number of environmental factors that could be involved. A possible way to tackle these diverse problems could be to consider highly associated comorbid conditions such as Gastro Intestinal Symptoms (GIS) along with the typical symptoms of the pathology.

Gastro Intestinal Symptoms (GIS) are a commonly identified co-morbidity in autistic children. Moreover, the presence of GIS has been reported to increase the severity of ASD behavior. Therefore, in order to overcome the severe behavioral symptoms of ASD, the in time diagnosis and treatment of GIS is crucial. These GIS point to the gut microbiota as a promising candidate in the manifestation of the pathophysiology of ASD.

The gut microbiota refers to the microbes that inhabit the human gut. These gut microbes live in the human gut by showing a symbiotic relationship, and performing certain crucial physiological roles in the human body. The timeframe for the development and maturation of central nervous system, and the formation, and stabilization of gut microbiota coincides. In addition to it, variations in gut microbial composition in ASD individuals as compared to normally developing ones have been reported in various studies. This opens up new avenues in ASD research by exploring gut microbiota and linking their association with ASD.

The microbiota-gut-brain axis marks the link of connection for the exchange of information between the microbiota, the gut, and the brain. The alterations in gut microbiota imparts negative effects on the brain development, and physiology through gut-brain axis which is the bidirectional mode of communication that exists between the gut microbes and Central Nervous System (CNS).

The bidirectional communication pathways between gut microbes and CNS are proposed to be neuroendocrine, autonomic nervous, toxins production, immunological and metabolic systems. The mode of effect of gut microbiota on brain development and physiology seems to an attractive and worthy perspective to explore the etiology.

Various studies have provided evidence of the pivotal role played by the gut microbial diversity in onset and variations in symptoms. The idea is further endorsed by the fact that symptoms are relieved in patients of ASD receiving prebiotics and probiotics, and fecal microbiota transplantation ameliorates the autistic behavior. Despite these evidences, the mechanisms by which the microbiota can possibly cause ASD, or associate with ASD symptoms is not yet clear. Thus, exploring the alterations in gut microbiota and their metabolites as well as their association with ASD symptoms could help in better understanding of ASD etiology.

In addition to genetic components microbial metabolites could be used in better and efficient diagnosis as well as treatment and management strategies.Several studies have been conducted to analyze the microbial composition in ASD subjects and the results obtained by different studies are inconsistent. Various studies have shown the variations in the composition of gut microbiota in ASD children when compared to their siblings, or other NT developing children.

The results obtained by different research groups to assess the gut microbial composition of ASD subjects as compared to their siblings or neurotypically developing children are inconsistent and no conclusive findings can be drawn but owing to the changes and variations in gut microbial composition, and impact of gut microbes in human health, it is provoking to explore this area more extensively to better underpin the etiology of ASD.

As no elucidation of the pathways and mechanisms by which these microbes add to ASD exists, we need to quest for the gut microbial metabolites and their role in association with the onset or the underlying pathophysiology of the disorder. These gut microbial metabolites could further be used for early diagnosis so that the financial, and emotional disease burden could be reduced.

This study was conducted in order to understand and elaborate the Microbiota-Gut-Brain Axis alterations in ASD. The assessment of variations in gut microbiota in ASD individuals was carried out in the first module of the research. Meta-analysis was carried out for this purpose by exploring the electronic databases till July 2023,

and computing relative abundance through Revman 5.3. The results depicted higher relative abundance of *Clostridium* and *Faecalibacterium*, and lower relative abundance of *Bifidobacterium* and *Coprococcus* in ASD children as opposed to healthy controls.

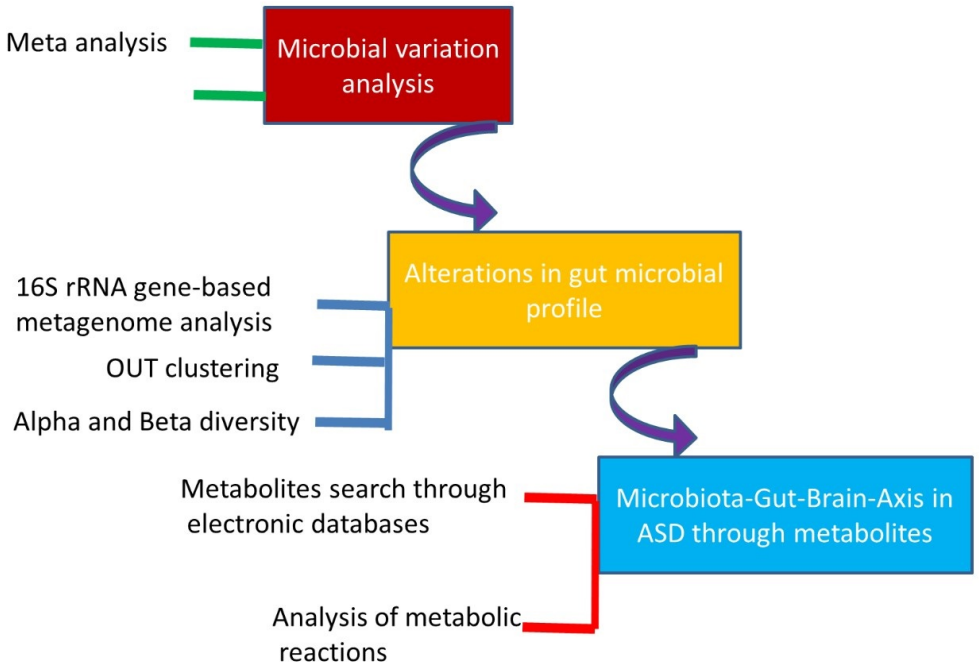

FIGURE 6.2: Various methodological steps in the research project.

The second module of the research revolved around the assessment of gut microbial diversity in ASD children. To execute the project, 16S rRNA sequencing based metagenome analysis was carried out by enrolling two autistic children and two neurotypically growing ones. The results of the metagenome analysis after OTU clustering showed the statistical differential variation of Lachnospiraceae UCG-004 genera in ASD children as compared to the neurotypically growing ones. The outcome of alpha diversity exhibited the lower specie diversity and richness in ASD children. The beta diversity delineated the differential clustering in autistic and control group.

The third and final objective of the study was to underpin the microbiota-gut-brain axis through metabolites. In order to proceed for this step, the metabolites produced from the various species of the genus that were identified and screened in the objective one, and two were searched through electronic databases. L-Acetone, D-lactic acid, Vinylacetyl CoA, and 10-formyltetrahydrofolate were identified to be produced by

various specie of genera screened by the current research project through meta-analysis and metagenome analysis.

Afterwards, the metabolic reactions of identified metabolites was carried out through Metaboanalyst. These metabolites are involved in several pathways such as ketone bodies metabolisum, gut microbial disbiosis, fatty acid metabolisum, and folate metabolisum pathway.

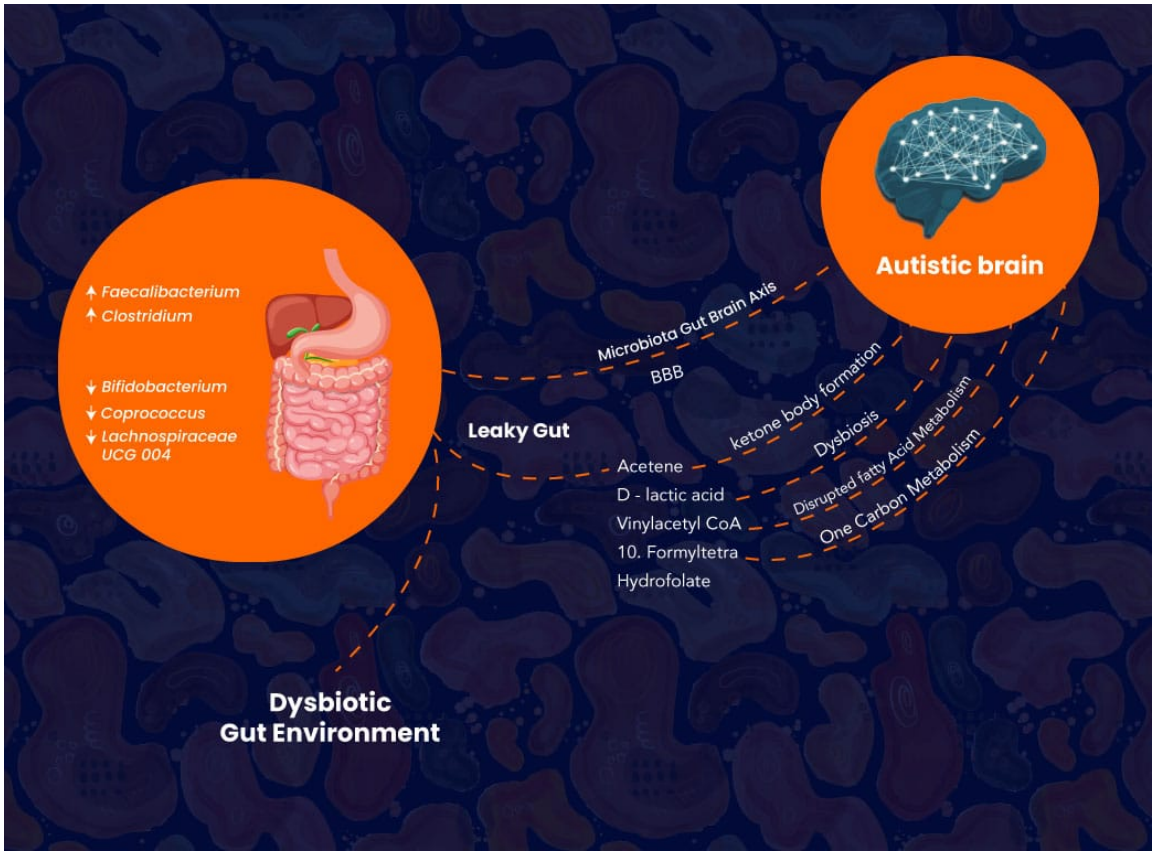

FIGURE 6.3: Possible implications of gut microbiota in ASD.

ASD being a disorder with various etiological factors is complex as well as obscure, and affects one in 36 children globally. The multifactorial nature halts the early diagnosis and lesser treatment approaches are offered to the individuals. Thus, it has been a great thirst for scientific researchers to plunge in to the various factors that could potentially play their part in ASD. Gut microbiota has been established to be involved in ASD. However, the exact mechanisms and pathways of involvement are yet to be discovered. The current study analyzed the gut microbial variations in ASD individuals as compared to the normally developed ones. The results obtained

add on in the repository of ASD related research work, and intrigue the researchers to further explore the gut microbial pathways and reactions that could be involved in the pathophysiology of ASD. The relative abundance beneficial bacteria like bifidobacterium, coprococcus, and lachnospiracece ucg004 has been found to be lower in ASD individuals and these genera could be given as probiotics for better therapeutic interventions.

6.2 Future Work and Recommendations

Future research on Autism Spectrum Disorder (ASD) should prioritize a comprehensive examination of the pathological mechanisms involved, with a particular emphasis on the gut-brain axis. The gut-brain axis represents a critical area of investigation due to its significant influence on neurodevelopment and behavior. Understanding the bidirectional interactions between the gastrointestinal system and the central nervous system could reveal new insights into ASD pathophysiology.

To advance our understanding of ASD, future studies should focus on the detailed exploration of metabolite interactions within the context of the disorder. Metabolomics, the study of metabolites, offers a promising avenue for identifying specific biochemical pathways and potential biomarkers involved in ASD. By leveraging advanced analytical techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy, researchers can characterize the metabolomic profiles of ASD individuals.

This approach may uncover novel metabolic signatures associated with ASD, leading to improved diagnostic tools and therapeutic strategies. Additionally, it is essential to investigate the interplay between genetic and environmental factors in the etiology of ASD. Genetic studies should aim to identify novel risk genes and elucidate their functional roles in neurodevelopment. Integrative approaches, such as genome-wide association studies (GWAS) and whole-genome sequencing, can provide valuable insights into the genetic architecture of ASD. Concurrently, environmental research should focus on identifying and characterizing prenatal and early-life exposures that

contribute to ASD risk. This includes examining the effects of maternal nutrition, infections, and environmental toxins on fetal brain development.

To facilitate a holistic understanding of ASD, interdisciplinary collaborations are recommended. Combining expertise from fields such as genetics, neuroscience, microbiology, and environmental science can lead to a more comprehensive understanding of the disorder. Longitudinal cohort studies and multi-omics approaches, which integrate genomics, transcriptomics, proteomics, and metabolomics, are particularly valuable for capturing the complex interactions underlying ASD.

In summary, future research on ASD should prioritize:

- Detailed exploration of the gut-brain axis and its implications in ASD pathophysiology.
- Comprehensive metabolomic studies to identify potential biomarkers and therapeutic targets.
- Investigation of the interplay between genetic and environmental factors in ASD etiology.
- Interdisciplinary collaborations to facilitate a holistic understanding of the disorder.
- Utilization of advanced analytical and integrative approaches to capture the complexity of ASD.

By addressing these recommendations, researchers can enhance our understanding of ASD and contribute to the development of targeted interventions and therapies, ultimately improving outcomes for individuals with ASD and their families.

Bibliography

[1] E. Garcia-Gutierrez, A. Narbad, and J. M. Rodriguez. Autism spectrum disorder associated with gut microbiota at immune, metabolomic, and neuroactive level. *Frontiers in neuroscience*, 14:578666, 2020. doi: 10.3389/fnins.2020.578666.

[2] M. J. Maenner, Z. Warren, A. R. Williams, E. Amoakohene, A. V. Bakian, D. A. Bilder, M. S. Durkin, R. T. Fitzgerald, S. M. Furnier, M. M. Hughes, C. M. Ladd-Acosta, D. McArthur, E. T. Pas, A. Salinas, A. Vehorn, S. Williams, A. Esler, A. Grzybowski, J. Hall-Lande, R. H. N. Nguyen, others, and K. A. Shaw. Prevalence and characteristics of autism spectrum disorder among children aged 8 years - autism and developmental disabilities monitoring network, 11 sites, united states. *2020. Morbidity and mortality weekly report. Surveillance summaries (Washington, D. C.*, 72(2):2002, 2023. doi: 10.15585/mmwr.ss7202a1.

[3] P. McCarty and R. E. Frye. Early detection and diagnosis of autism spectrum disorder: Why is it so difficult? *Seminars in pediatric neurology*, 35:100831, 2020. doi: 10.1016/j.spen.2020.100831.

[4] A. Genovese and M. G. Butler. Clinical assessment, genetics, and treatment approaches in autism spectrum disorder (asd). *International journal of molecular sciences*, 21(13):4726, 2020. doi: 10.3390/ijms21134726.

[5] C. Lord, T. S. Brugha, T. Charman, J. Cusack, G. Dumas, T. Frazier, E. J. H. Jones, R. M. Jones, A. Pickles, M. W. State, J. L. Taylor, and J. Veenstra-VanderWeele. Autism spectrum disorder. *Nature reviews. Disease primers*, 6 (1):5, 2020. doi: 10.1038/s41572-019-0138-4.

[6] G. Leader, C. Abberton, S. Cunningham, K. Gilmartin, M. Grudzien, E. Higgins, L. Joshi, S. Whelan, and A. Mannion. Gastrointestinal symptoms in autism spectrum disorder: A systematic review. *Nutrients*, 14:1471, 2022. doi: 10.3390/nu14071471.

[7] S. H. Zyoud, M. Shakhshir, A. S. Abushanab, A. Koni, M. Shahwan, A. A. Jairoun, A. Abu Taha, and S. W. Al-Jabi. Gut microbiota and autism spectrum disorders: where do we stand? *Gut pathogens*, 15(1):50, 2023. doi: 10.1186/s13099-023-00575-8.

[8] P. Srikantha and M. H. Mohajeri. The possible role of the microbiota-gut-brain-axis in autism spectrum disorder. *International journal of molecular sciences*, 20(9):2115, 2019. doi: 10.3390/ijms20092115.

[9] L. Iglesias-Vazquez, G. Van Ginkel Riba, V. Arija, and J. Canals. Composition of gut microbiota in children with autism spectrum disorder: A systematic review and meta-analysis. *Nutrients*, 12(3):792, 2020. doi: 10.3390/nu12030792.

[10] A. B. Haidich. Meta-analysis in medical research. *Hippokratia*, 14(Suppl 1): 29–37, 2010.

[11] J. H. Jo, E. A. Kennedy, and H. H. Kong. Research techniques made simple: Bacterial 16s ribosomal rna gene sequencing in cutaneous research. *The Journal of investigative dermatology*, 136(3):e23–e27, 2016. doi: 10.1016/j.jid.2016.01.005.

[12] R. Bharti and D. G. Grimm. Current challenges and best-practice protocols for microbiome analysis. *Briefings in bioinformatics*, 22(1):178–193, 2021. doi: 10.1093/bib/bbz155.

[13] Asmita Kamble, Shriya Sawant, and Harinder Singh. 16s ribosomal rna gene-based metagenomics: A review. *Biomedical Research Journal.*, 7:5, 2020. doi: 10.4103/BMRJ.BMRJ_4_20.

[14] Q. Wang and R. Xu. Data-driven multiple-level analysis of gut-microbiome-immune-joint interactions in rheumatoid arthritis. *BMC genomics*, 20(1):124, 2019. doi: 10.1186/s12864-019-5510-y.

[15] M. Kuhn, C. von Mering, M. Campillos, L. J. Jensen, and P. Bork. Stitch: interaction networks of chemicals and proteins. *Nucleic acids research*, 36(Database issue):D684–D688, 2008. doi: 10.1093/nar/gkm795.

[16] Hongbin Yang, Chaofeng Lou, Lixia Sun, Jie Li, Yingchun Cai, Zhuang Wang, Weihua Li, Guixia Liu, and Yun Tang admetSAR. 2.0: web-service for prediction and optimization of chemical admet properties. *Bioinformatics*, 35(6):1067–1069, March 2019.

[17] L. Wang, B. Wang, C. Wu, J. Wang, and M. Sun. Autism spectrum disorder: Neurodevelopmental risk factors, biological mechanism, and precision therapy. *International journal of molecular sciences*, 24(3):1819, 2023. doi: 10.3390/ijms24031819.

[18] Leo Kanner. Autistic disturbances of affective contact. *Nervous Child: Journal of Psychopathology, Psychotherapy, Mental Hygiene, and Guidance of the Child*, 2:217–50, 1943.

[19] N. Salari, S. Rasoulpoor, S. Rasoulpoor, S. Shohaimi, S. Jafarpour, N. Abdoli, B. Khaledi-Paveh, and M. Mohammadi. The global prevalence of autism spectrum disorder: a comprehensive systematic review and meta-analysis. *Italian journal of pediatrics*, 48(1):112, 2022. doi: 10.1186/s13052-022-01310-w.

[20] M. Khalid, H. Raza, T. M. Driessen, P. J. Lee, L. Tejwani, A. Sami, M. Nawaz, S. Mehmood Baig, J. Lim, and G. Kaukab Raja. Genetic risk of autism spectrum disorder in a pakistani population. *Genes*, 11(10):1206, 2020. doi: 10.3390/genes11101206.

[21] A. Masi, M. M. DeMayo, N. Glozier, and A. J. Guastella. An overview of autism spectrum disorder, heterogeneity and treatment options. *Neuroscience bulletin*, 33(2):183–193, 2017. doi: 10.1007/s12264-017-0100-y.

[22] American Psychiatric Association. *(APA) (2013) Diagnostic and Statistical Manual of Mental Disorders (DSM-V)*. American Psychiatric Publishing, Washington DC, 5th edition. doi: 10.1176/appi.books.9780890425596.

[23] P. McCarty and R. E. Frye. Early detection and diagnosis of autism spectrum disorder: Why is it so difficult? *Seminars in pediatric neurology*, 35:100831, 2020. doi: 10.1016/j.spen.2020.100831.

[24] Nicole Wolff, Sanna Stroth, Inge Kamp-Becker, Stefan Roepke, and Veit Roessner. Autism spectrum disorder and iq – a complex interplay. *Frontiers in Psychiatry.*, 13, 2022. doi: 10.3389/fpsyt.2022.856084.

[25] M. Micai, L. M. Fatta, L. Gila, A. Caruso, T. Salvitti, F. Fulceri, A. Ciaramella, R. D'Amico, C. Del Giovane, M. Bertelli, G. Romano, H. J. Schunemann, and M. L. Scattoni. *Prevalence of co-occurring conditions in children and adults with autism spectrum disorder: A systematic review and meta-analysis. Neuroscience and biobehavioral reviews, 155, 105, 436.* Co-occurring conditions, 2023. doi: 10.1016/j.neubiorev.2023.105436.

[26] A. Mackay, A. Burford, D. Carvalho, E. Izquierdo, J. Fazal-Salom, K. R. Taylor, L. Bjerke, M. Clarke, M. Vinci, M. Nandhabalan, S. Temelso, S. Popov, V. Molinari, P. Raman, A. J. Waanders, H. J. Han, S. Gupta, L. Marshall, S. Zacharoulis, S. Vaidya, others, and C. Jones. Integrated molecular meta-analysis of 1,000 pediatric high-grade and diffuse intrinsic pontine glioma. *Cancer cell*, 32(4):520–537, 2017. doi: 10.1016/j.ccell.2017.08.017.

[27] M. Hong, S. M. Lee, S. Park, S. J. Yoon, Y. E. Kim, and I. H. Oh. Prevalence and economic burden of autism spectrum disorder in south korea using national health insurance data from 2008 to 2015. *Journal of autism and developmental disorders*, 50(1):333–339, 2020. doi: 10.1007/s10803-019-04255-y.

[28] Y. Zhao, F. Lu, X. Wang, Y. Luo, R. Zhang, P. He, and X. Zheng. *The economic burden of autism spectrum disorder with and without intellectual disability in China: A nationwide cost-of-illness study. Asian journal of psychiatry, 92, 103877.* Advance online publication, 2023. doi: 10.1016/j.ajp.2023.103877.

[29] J. P. Leigh and J. Du. Brief report: Forecasting the economic burden of autism in 2015 and 2025 in the united states. *Journal of autism and developmental disorders*, 45(12):4135–4139, 2015. doi: 10.1007/s10803-015-2521-7.

[30] E. Pisula and A. Porebowicz-Dorsmann. Family functioning, parenting stress and quality of life in mothers and fathers of polish children with high functioning autism or asperger syndrome. *PloS one*, 12(10):e0186536, 2017. doi: 10.1371/journal.pone.0186536.

[31] K. Van Niekerk, V. Stancheva, and C. Smith. Caregiver burden among caregivers of children with autism spectrum disorder. *The South African journal of psychiatry : SAJP : the journal of the Society of Psychiatrists of South Africa*, 29:2079, 2023. doi: 10.4102/sajpsychiatry.v29i0.2079.

[32] S. M. Alkhonezan, M. M. Alkhonezan, Y. Alshayea, H. Bukhari, and R. Almhizai. Factors influencing the lives of parents of children with autism spectrum disorder in saudi arabia: A comprehensive review. *Cureus*, 15(11): e48325, 2023. doi: 10.7759/cureus.48325.

[33] Kieran A. Cook and Alissa N. Willmerdinger. The history of autism, 2015. URL http://scholarexchange.furman.edu/schopler-about/1. Narrative Documents. Book 1.

[34] I. A. Sindi. Implications of cell adhesion molecules in autism spectrum disorder pathogenesis. *Journal of microscopy and ultrastructure*, 11(4):199–205, 2022. doi: 10.4103/jmau.jmau_15_22.

[35] S. Qiu, Y. Qiu, Y. Li, and X. Cong. Genetics of autism spectrum disorder: an umbrella review of systematic reviews and meta-analyses. *Translational psychiatry*, 12(1):249, 2022. doi: 10.1038/s41398-022-02009-6.

[36] E. Masini, E. Loi, A. F. Vega-Benedetti, M. Carta, G. Doneddu, R. Fadda, and P. Zavattari. An overview of the main genetic, epigenetic and environmental factors involved in autism spectrum disorder focusing on synaptic activity. *International journal of molecular sciences*, 21(21):8290, 2020. doi: 10.3390/ijms21218290.

[37] S. Bolte, S. Girdler, and P. B. Marschik. The contribution of environmental exposure to the etiology of autism spectrum disorder. *Cellular and molecular life sciences : CMLS*, 76(7):1275–1297, 2019. doi: 10.1007/s00018-018-2988-4.

[38] A. A. Eshraghi, G. Liu, S. S. Kay, R. S. Eshraghi, J. Mittal, B. Moshiree, and R. Mittal. Epigenetics and autism spectrum disorder: Is there a correlation? *Frontiers in cellular neuroscience*, 12:78, 2018. doi: 10.3389/fncel.2018.00078.

[39] P. Y. Pan, M. J. Taylor, H. Larsson, C. Almqvist, P. Lichtenstein, S. Lundstrom, and S. Bolte. Genetic and environmental contributions to co-occurring physical health conditions in autism spectrum condition and attention-deficit/hyperactivity disorder. *Molecular autism*, 14(1):17, 2023. doi: 10.1186/s13229-023-00548-3.

[40] G. Leader, C. Abberton, S. Cunningham, K. Gilmartin, M. Grudzien, E. Higgins, L. Joshi, S. Whelan, and A. Mannion. Gastrointestinal symptoms in autism spectrum disorder: A systematic review. *Nutrients*, 14(7):1471, 2022. doi: 10.3390/nu14071471.

[41] M. Madra, R. Ringel, and K. G. Margolis. Gastrointestinal issues and autism spectrum disorder. *Child and adolescent psychiatric clinics of North America*, 29(3):501–513, 2020. doi: 10.1016/j.chc.2020.02.005.

[42] M. Al-Beltagi, N. K. Saeed, A. S. Bediwy, R. Elbeltagi, and R. Alhawamdeh. Role of gastrointestinal health in managing children with autism spectrum disorder. *World journal of clinical pediatrics*, 12(4):171–196, 2023. doi: 10.5409/wjcp.v12.i4.171.

[43] Ruchika Kalra, Meena Gupta, and Priya Sharma. Recent advancement in interventions for autism spectrum disorder: A review. *Journal of Neurorestoratology.*, 11:100068, 2023. doi: 10.1016/j.jnrt.2023.100068.

[44] S. J. Elliott, D. Marshall, K. Morley, E. Uphoff, M. Kumar, and N. Meader. Behavioural and cognitive behavioural therapy for obsessive compulsive disorder (ocd) in individuals with autism spectrum disorder (asd). *The Cochrane*

database of systematic reviews, 9(9):CD013173, 2021. doi: 10.1002/14651858.CD013173.pub2.

[45] S. Siafis, O. o ray, H. Wu, J. Schneider-Thoma, I. Bighelli, M. Krause, A. Rodolico, A. Ceraso, G. Deste, M. Huhn, D. Fraguas, A. San Joso Caceres, D. Mavridis, T. Charman, D. G. Murphy, M. Parellada, C. Arango, and S. Leucht. Pharmacological and dietary-supplement treatments for autism spectrum disorder: a systematic review and network meta-analysis. *Molecular autism*, 13(1):10, 2022. doi: 10.1186/s13229-022-00488-4.

[46] C. Lord, M. Elsabbagh, G. Baird, and J. Veenstra-Vanderweele. Autism spectrum disorder. lancet (london. *England)*, 392(10146):508–520, 2018. doi: 10.1016/S0140-6736(18)31129-2.

[47] R. Aishworiya, T. Valica, R. Hagerman, and B. Restrepo. An update on psychopharmacological treatment of autism spectrum disorder. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*, 19 (1):248–262, 2022. doi: 10.1007/s13311-022-01183-1.

[48] A. Mehra, G. Arora, G. Sahni, M. Kaur, H. Singh, B. Singh, and S. Kaur. Gut microbiota and autism spectrum disorder: From pathogenesis to potential therapeutic perspectives. *Journal of traditional and complementary medicine*, 13(2):135–149, 2022. doi: 10.1016/j.jtcme.2022.03.001.

[49] J. Zhang, G. Zhu, L. Wan, Y. Liang, X. Liu, H. Yan, B. Zhang, and G. Yang. Effect of fecal microbiota transplantation in children with autism spectrum disorder: A systematic review. *Frontiers in psychiatry*, 14, 2023. doi: 10.3389/fpsyt.2023.1123658. Article 1123658.

[50] R. Sender, S. Fuchs, and R. Milo. Revised estimates for the number of human and bacteria cells in the body. *PLoS biology*, 14(8):e1002533, 2016. doi: 10.1371/journal.pbio.1002533.

[51] J. Liu, Z. Gao, C. Liu, T. Liu, J. Gao, Y. Cai, and X. Fan. Alteration of gut microbiota: New strategy for treating autism spectrum disorder. *Frontiers in cell and developmental biology*, 10:792490, 2022. doi: 10.3389/fcell.2022.792490.

[52] V. N. Dargenio, C. Dargenio, S. Castellaneta, A. De Giacomo, M. Laguardia, F. Schettini, R. Francavilla, and F. Cristofori. Intestinal barrier dysfunction and microbiota-gut-brain axis: Possible implications in the pathogenesis and treatment of autism spectrum disorder. *Nutrients*, 15(7):1620, 2023. doi: 10.3390/nu15071620.

[53] K. Gawli-ska, D. Gawli-ski, M. Borczyk, M. Korosty-ski, E. Przegali-ski, and M. Filip. A maternal high-fat diet during early development provokes molecular changes related to autism spectrum disorder in the rat offspring brain. *Nutrients*, 13(9):3212, 2021. doi: 10.3390/nu13093212.

[54] C. Crump, J. Sundquist, and K. Sundquist. Preterm or early term birth and risk of autism. *Pediatrics*, 148(3):e2020032300, 2021. doi: 10.1542/peds.2020-032300.

[55] A. H. Al-Zalabani, A. H. Al-Jabree, and Z. A. Zeidan. Is cesarean section delivery associated with autism spectrum disorder? *Neurosciences (Riyadh, Saudi Arabia)*, 24(1):11–15, 2019. doi: 10.17712/nsj.2019.1.20180303.

[56] K. Y. Liu, J. O. Teitler, S. Rajananda, V. Chegwin, P. S. Bearman, T. Hegyi, and N. E. Reichman. Elective deliveries and the risk of autism. *Am J Prev Med*, 63:68–76, 2022.

[57] E. Jenabi, S. Bashirian, A. M. Salehi, and S. Khazaei. Not breastfeeding and risk of autism spectrum disorders among children: a meta-analysis. *Clinical and experimental pediatrics*, 66(1):28–31, 2023. doi: 10.3345/cep.2021.01872.

[58] L. L. Njotto, J. Simin, R. Fornes, I. Odsbu, I. Mussche, S. Callens, L. Engstrand, R. Bruyndonckx, and N. Brusselaers. Maternal and early-life exposure to antibiotics and the risk of autism and attention-deficit hyperactivity disorder in childhood: a swedish population-based cohort study. *Drug safety*, 46(5):467–478, 2023. doi: 10.1007/s40264-023-01297-1.

[59] A. Zawadzka, M. Cie-lik, and A. Adamczyk. The role of maternal immune activation in the pathogenesis of autism: A review of the evidence, proposed

mechanisms and implications for treatment. *International journal of molecular sciences*, 22(21):11516, 2021. doi: 10.3390/ijms222111516.

[60] I. Lasheras, P. Seral, E. Latorre, E. Barroso, P. Gracia-Garcia, and J. Santabarbara. Microbiota and gut-brain axis dysfunction in autism spectrum disorder: Evidence for functional gastrointestinal disorders. *Asian journal of psychiatry*, 47:101874, 2020. doi: 10.1016/j.ajp.2019.101874.

[61] F. Li, H. Ke, S. Wang, W. Mao, C. Fu, X. Chen, Q. Fu, X. Qin, Y. Huang, B. Li, S. Li, J. Xing, M. Wang, and W. Deng. Leaky gut plays a critical role in the pathophysiology of autism in mice by activating the lipopolysaccharide-mediated toll-like receptor 4-myeloid differentiation factor 88-nuclear factor kappa b signaling pathway. *Neuroscience bulletin*, 39(6):911–928, 2023. doi: 10.1007/s12264-022-00993-9.

[62] V. N. Dargenio, C. Dargenio, S. Castellaneta, A. De Giacomo, M. Laguardia, F. Schettini, R. Francavilla, and F. Cristofori. Intestinal barrier dysfunction and microbiota-gut-brain axis: Possible implications in the pathogenesis and treatment of autism spectrum disorder. *Nutrients*, 15(7):1620, 2023. doi: 10.3390/nu15071620.

[63] C. Piras, M. Mussap, A. Noto, A. De Giacomo, F. Cristofori, M. Spada, V. Fanos, L. Atzori, and R. Francavilla. Alterations of the intestinal permeability are reflected by changes in the urine metabolome of young autistic children: Preliminary results. *Metabolites*, 12(2):104, 2022. doi: 10.3390/metabo12020104.

[64] A. Camasio, E. Panzeri, L. Mancuso, T. Costa, J. Manuello, M. Ferraro, S. Duca, F. Cauda, and D. Liloia. Linking neuroanatomical abnormalities in autism spectrum disorder with gene expression of candidate asd genes: A meta-analytic and network-oriented approach. *PloS one*, 17(11):e0277466, 2022. doi: 10.1371/journal.pone.0277466.

[65] A. Beopoulos, M. Goa, A. Fasano, and F. Iris. Autism spectrum disorders pathogenesis: Toward a comprehensive model based on neuroanatomic and neurodevelopment considerations. *Frontiers in neuroscience*, 16:988735, 2022. doi: 10.3389/fnins.2022.988735.

[66] A. Alharthi, S. Alhazmi, N. Alburae, and A. Bahieldin. The human gut microbiome as a potential factor in autism spectrum disorder. *International journal of molecular sciences*, 23(3):1363, 2022. doi: 10.3390/ijms23031363.

[67] G. Makris, A. Agorastos, G. P. Chrousos, and P. Pervanidou. Stress system activation in children and adolescents with autism spectrum disorder. *Frontiers in neuroscience*, 15:756628, 2022. doi: 10.3389/fnins.2021.756628.

[68] F. Zhao, H. Zhang, P. Wang, W. Cui, K. Xu, D. Chen, M. Hu, Z. Li, X. Geng, and S. Wei. Oxytocin and serotonin in the modulation of neural function: Neurobiological underpinnings of autism-related behavior. *Frontiers in neuroscience*, 16:919890, 2022. doi: 10.3389/fnins.2022.919890.

[69] R. M. Lungba, S. Z. A. Khan, U. Ajibawo-Aganbi, et al. The role of the gut microbiota and the immune system in the development of autism. *Cureus.*, 12 (10):e11226, 2020.

[70] A. De Sales-Millan, J. F. Aguirre-Garrido, R. M. Gonzalez-Cervantes, and J. A. Velazquez-Aragon. Microbiome–gut–mucosal–immune–brain axis and autism spectrum disorder (asd): A novel proposal of the role of the gut microbiome in asd aetiology. *Behavioural science.*, 13:548, 2023. doi: 10.3390/bs13070548.

[71] A. Meltzer and J. Van de Water. The role of the immune system in autism spectrum disorder. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 42(1):284–298, 2017. doi: 10.1038/npp.2016.158.

[72] M. L. A. Robinson-Agramonte, E. Noris Garcia, J. Fraga Guerra, Y. Vega Hurtado, N. Antonucci, N. Sempron-Hernandez, S. Schultz, and D. Siniscalco. Immune dysregulation in autism spectrum disorder: What do we know about

it? *International journal of molecular sciences*, 23(6):3033, 2022. doi: 10.3390/ijms23063033.

[73] A. Erbescu, S. M. Papuc, M. Budisteanu, A. Arghir, and M. Neagu. Re-emerging concepts of immune dysregulation in autism spectrum disorders. *Frontiers in psychiatry*, 13, 2022. doi: 10.3389/fpsyt.2022.1006612. Article 1006612.

[74] S. Gyawali and B. N. Patra. Autism spectrum disorder: Trends in research exploring etiopathogenesis. *Psychiatry and clinical neurosciences*, 73(8):466–475, 2019. doi: 10.1111/pcn.12860.

[75] H. Sabit, H. Tombuloglu, S. Rehman, N. B. Almandil, E. Cevik, S. Abdel-Ghany, S. Rashwan, M. F. Abasiyanik, and M. M. Yee Waye. Gut microbiota metabolites in autistic children: An epigenetic perspective. *Heliyon*, 7(1):e06105, 2021. doi: 10.1016/j.heliyon.2021.e06105.

[76] V. Saurman, K. G. Margolis, and R. A. Luna. Autism spectrum disorder as a brain-gut-microbiome axis disorder. *Digestive diseases and sciences*, 65(3): 818–828, 2020. doi: 10.1007/s10620-020-06133-5.

[77] D. Brister, S. Rose, L. Delhey, M. Tippett, Y. Jin, H. Gu, and R. E. Frye. Metabolomic signatures of autism spectrum disorder. *Journal of personalized medicine*, 12(10):1727, 2022. doi: 10.3390/jpm12101727.

[78] S. M. Finegold, D. Molitoris, Y. Song, C. Liu, M. L. Vaisanen, E. Bolte, M. McTeague, R. Sandler, H. Wexler, E. M. Marlowe, M. D. Collins, P. A. Lawson, P. Summanen, M. Baysallar, T. J. Tomzynski, E. Read, E. Johnson, R. Rolfe, P. Nasir, H. Shah, others, and A. Kaul. Gastrointestinal microflora studies in late-onset autism. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 35(Suppl 1):S6–S16, 2002. doi: 10.1086/341914.

[79] Y. Song, C. Liu, and S. M. Finegold. Real-time pcr quantitation of clostridia in feces of autistic children. *Applied and environmental microbiology*, 70(11): 6459–6465, 2004. doi: 10.1128/AEM.70.11.6459-6465.2004.

[80] H. M. Parracho, M. O. Bingham, G. R. Gibson, and A. L. McCartney. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *Journal of medical microbiology*, 54(Pt 10):987–991, 2005. doi: 10.1099/jmm.0.46101-0.

[81] J. B. Adams, L. J. Johansen, L. D. Powell, D. Quig, and R. A. Rubin. Gastrointestinal flora and gastrointestinal status in children with autism–comparisons to typical children and correlation with autism severity. *BMC gastroenterology*, 11: 22, 2011. doi: 10.1186/1471-230X-11-22.

[82] L. Wang, C. T. Christophersen, M. J. Sorich, J. P. Gerber, M. T. Angley, and M. A. Conlon. Low relative abundances of the mucolytic bacterium akkermansia muciniphila and bifidobacterium spp. in feces of children with autism. *Applied and environmental microbiology*, 77(18):6718–6721, 2011. doi: 10.1128/AEM.05212-11.

[83] B. L. Williams, M. Hornig, T. Buie, M. L. Bauman, M. Cho Paik, I. Wick, A. Bennett, O. Jabado, D. L. Hirschberg, and W. I. Lipkin. Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. *PloS one*, 6(9):e24585, 2011. doi: 10.1371/journal.pone.0024585.

[84] B. L. Williams, M. Hornig, T. Parekh, and W. I. Lipkin. Application of novel pcr-based methods for detection, quantitation, and phylogenetic characterization of sutterella species in intestinal biopsy samples from children with autism and gastrointestinal disturbances. *Medical Biology*, 3(1):e00261–11, 2012. doi: 10.1128/mBio.00261-11.

[85] S. V. Gondalia, E. A. Palombo, S. R. Knowles, S. B. Cox, D. Meyer, and D. W. Austin. Molecular characterisation of gastrointestinal microbiota of children with autism (with and without gastrointestinal dysfunction) and their neurotypical siblings. *Autism Research : Official Journal of the International Society for Autism Research*, 5(6):419–427, 2012. doi: 10.1002/aur.1253.

[86] L. Wang, C. T. Christophersen, M. J. Sorich, J. P. Gerber, M. T. Angley, and M. A. Conlon. Increased abundance of sutterella spp. and ruminococcus torques in feces of children with autism spectrum disorder. *Molecular autism*, 4(1):42, 2013. doi: 10.1186/2040-2392-4-42.

[87] D. W. Kang, J. G. Park, Z. E. Ilhan, G. Wallstrom, J. Labaer, J. B. Adams, and R. Krajmalnik-Brown. Reduced incidence of prevotella and other fermenters in intestinal microflora of autistic children. *PloS one*, 8(7):e68322, 2013. doi: 10.1371/journal.pone.0068322.

[88] M. De Angelis, M. Piccolo, L. Vannini, S. Siragusa, A. De Giacomo, D. I. Serrazzanetti, F. Cristofori, M. E. Guerzoni, M. Gobbetti, and R. Francavilla. Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PloS one*, 8(10):e76993, 2013. doi: 10.1371/journal.pone.0076993.

[89] A. Tomova, V. Husarova, S. Lakatosova, J. Bakos, B. Vlkova, K. Babinska, and D. Ostatnikova. Gastrointestinal microbiota in children with autism in slovakia. *Physiology and behavior*, 138:179–187, 2015. doi: 10.1016/j.physbeh.2014.10.033.

[90] R. Inoue, Y. Sakaue, C. Sawai, T. Sawai, M. Ozeki, G. A. Romero-Porez, and T. Tsukahara. A preliminary investigation on the relationship between gut microbiota and gene expressions in peripheral mononuclear cells of infants with autism spectrum disorders. *Bioscience, biotechnology, and biochemistry*, 80(12):2450–2458, 2016. doi: 10.1080/09168451.2016.1222267.

[91] J. S. Son, L. J. Zheng, L. M. Rowehl, X. Tian, Y. Zhang, W. Zhu, L. Litcher-Kelly, K. D. Gadow, G. Gathungu, C. E. Robertson, D. Ir, D. N. Frank, and E. Li. Comparison of fecal microbiota in children with autism spectrum disorders and neurotypical siblings in the simons simplex collection. *PloS one*, 10(10):e0137725, 2015. doi: 10.1371/journal.pone.0137725.

[92] M. R. Iovene, F. Bombace, R. Maresca, A. Sapone, P. Iardino, A. Picardi, R. Marotta, C. Schiraldi, D. Siniscalco, N. Serra, L. de Magistris, and C. Bravaccio. Intestinal dysbiosis and yeast isolation in stool of subjects with autism spectrum disorders. *Mycopathologia*, 182(3-4):349–363, 2017. doi: 10.1007/s11046-016-0068-6.

[93] S. M. Finegold, P. H. Summanen, J. Downes, K. Corbett, and T. Komoriya. Detection of clostridium perfringens toxin genes in the gut microbiota of autistic children. *Anaerobe*, 45:133–137, 2017. doi: 10.1016/j.anaerobe.2017.02.008.

[94] F. Strati, D. Cavalieri, D. Albanese, C. De Felice, C. Donati, J. Hayek, O. Jousson, S. Leoncini, D. Renzi, A. Calabro, and C. De Filippo. New evidences on the altered gut microbiota in autism spectrum disorders. *Microbiome*, 5(1):24, 2017. doi: 10.1186/s40168-017-0242-1.

[95] R. I. Kushak, H. S. Winter, T. M. Buie, S. B. Cox, C. D. Phillips, and N. L. Ward. Analysis of the duodenal microbiome in autistic individuals: Association with carbohydrate digestion. *Journal of pediatric gastroenterology and nutrition*, 64(5):e110–e116, 2017. doi: 10.1097/MPG.0000000000001458.

[96] Y. Lee, J. Y. Park, E. H. Lee, J. Yang, B. R. Jeong, Y. K. Kim, J. Y. Seoh, S. Lee, P. L. Han, and E. J. Kim. Rapid assessment of microbiota changes in individuals with autism spectrum disorder using bacteria-derived membrane vesicles in urine. *Experimental neurobiology*, 26(5):307–317, 2017. doi: 10.5607/en.2017.26.5.307.

[97] R. A. Luna, N. Oezguen, M. Balderas, A. Venkatachalam, J. K. Runge, J. Versalovic, J. Veenstra-VanderWeele, G. M. Anderson, T. Savidge, and K. C. Williams. Distinct microbiome-neuroimmune signatures correlate with functional abdominal pain in children with autism spectrum disorder. *Cellular and molecular gastroenterology and hepatology*, 3(2):218–230, 2016. doi: 10.1016/j.jcmgh.2016.11.008.

[98] D. W. Kang, Z. E. Ilhan, N. G. Isern, D. W. Hoyt, D. P. Howsmon, M. Shaffer, C. A. Lozupone, J. Hahn, J. B. Adams, and R. Krajmalnik-Brown. Differences

in fecal microbial metabolites and microbiota of children with autism spectrum disorders. *Anaerobe*, 49:121–131, 2018. doi: 10.1016/j.anaerobe.2017.12.007.

[99] M. Zhang, W. Ma, J. Zhang, Y. He, and J. Wang. Analysis of gut microbiota profiles and microbe-disease associations in children with autism spectrum disorders in China. *Scientific reports*, 8(1):13981, 2018. doi: 10.1038/s41598-018-32219-2.

[100] D. R. Rose, H. Yang, G. Serena, C. Sturgeon, B. Ma, M. Careaga, H. K. Hughes, K. Angkustsiri, M. Rose, I. Hertz-Picciotto, J. Van de Water, R. L. Hansen, J. Ravel, A. Fasano, and P. Ashwood. Differential immune responses and microbiota profiles in children with autism spectrum disorders and co-morbid gastrointestinal symptoms. *Brain, behavior, and immunity*, 70:354–368, 2018. doi: 10.1016/j.bbi.2018.03.025.

[101] B. Ma, J. Liang, M. Dai, J. Wang, J. Luo, Z. Zhang, and J. Jing. Altered gut microbiota in chinese children with autism spectrum disorders. *Frontiers in cellular and infection microbiology*, 9:40, 2019. doi: 10.3389/fcimb.2019.00040.

[102] S. Liu, E. Li, Z. Sun, D. Fu, G. Duan, M. Jiang, Y. Yu, L. Mei, P. Yang, Y. Tang, and P. Zheng. Altered gut microbiota and short chain fatty acids in chinese children with autism spectrum disorder. *Scientific reports*, 9(1):287, 2019. doi: 10.1038/s41598-018-36430-z.

[103] M. Wang, J. Wan, H. Rong, F. He, H. Wang, J. Zhou, C. Cai, Y. Wang, R. Xu, Z. Yin, and W. Zhou. Alterations in gut glutamate metabolism associated with changes in gut microbiota composition in children with autism spectrum disorder. msystems. 4(1):e00321–18, 2019. doi: 10.1128/mSystems.00321-18.

[104] J. Plaza-Diaz, A. Gomez-Fernandez, N. Chueca, M. J. Torre-Aguilar, a. Gil, J. L. Perez-Navero, K. Flores-Rojas, P. Martin-Borreguero, P. Solis-Urra, F. J. Ruiz-Ojeda, F. Garcia, and M. Gil-Campos. Autism spectrum disorder (asd) with and without mental regression is associated with changes in the fecal microbiota. *Nutrients*, 11(2):337, 2019. doi: 10.3390/nu11020337.

[105] X. Kong, J. Liu, M. Cetinbas, R. Sadreyev, M. Koh, H. Huang, A. Adeseye, P. He, J. Zhu, H. Russell, C. Hobbie, K. Liu, and A. B. Onderdonk. New

and preliminary evidence on altered oral and gut microbiota in individuals with autism spectrum disorder (asd): Implications for asd diagnosis and subtyping based on microbial biomarkers. *Nutrients*, 11(9):2128, 2019. doi: 10.3390/nu11092128.

[106] H. Sun, Z. You, L. Jia, and F. Wang. Autism spectrum disorder is associated with gut microbiota disorder in children. *BMC pediatrics*, 19(1):516, 2019. doi: 10.1186/s12887-019-1896-6.

[107] M. F. Zurita, P. A. Cardenas, M. E. Sandoval, M. C. Peoa, M. Fornasini, N. Flores, M. H. Monaco, K. Berding, S. M. Donovan, T. Kuntz, J. A. Gilbert, and M. E. Baldeon. Analysis of gut microbiome, nutrition and immune status in autism spectrum disorder: a case-control study in ecuador. *Gut microbes*, 11 (3):453–464, 2020. doi: 10.1080/19490976.2019.1662260.

[108] S. Hazan, K. D. Spradling-Reeves, A. Papoutsis, and S. J. Walker. Shotgun metagenomic sequencing identifies dysbiosis in triplet sibling with gastrointestinal symptoms and asd. children (basel. *Metagenome*, 7(12):255, 2020. doi: 10.3390/children7120255.

[109] X. Hua, J. Zhu, T. Yang, M. Guo, Q. Li, J. Chen, and T. Li. The gut microbiota and associated metabolites are altered in sleep disorder of children with autism spectrum disorders. *Frontiers in psychiatry*, 11:855, 2020. doi: 10.3389/fpsyt.2020.00855.

[110] Z. Dan, X. Mao, Q. Liu, M. Guo, Y. Zhuang, Z. Liu, K. Chen, J. Chen, R. Xu, J. Tang, L. Qin, B. Gu, K. Liu, C. Su, F. Zhang, Y. Xia, Z. Hu, and X. Liu. Altered gut microbial profile is associated with abnormal metabolism activity of autism spectrum disorder. *Gut microbes*, 11(5):1246–1267, 2020. doi: 10.1080/19490976.2020.1747329.

[111] H. Ding, X. Yi, X. Zhang, H. Wang, H. Liu, and W. W. Mou. Imbalance in the gut microbiota of children with autism spectrum disorders. *Frontiers in cellular and infection microbiology*, 11:572752, 2021. doi: 10.3389/fcimb.2021.572752.

[112] S. C. Fu, C. H. Lee, and H. Wang. Exploring the association of autism spectrum disorders and constipation through analysis of the gut microbiome. *International journal of environmental research and public health*, 18(2):667, 2021. doi: 10.3390/ijerph18020667.

[113] F. Ye, X. Gao, Z. Wang, S. Cao, G. Liang, D. He, Z. Lv, L. Wang, P. Xu, and Q. Zhang. Comparison of gut microbiota in autism spectrum disorders and neurotypical boys in china: A case-control study. *Synthetic and systems biotechnology*, 6(2):120–126, 2021. doi: 10.1016/j.synbio.2021.03.003.

[114] X. Ding, Y. Xu, X. Zhang, L. Zhang, G. Duan, C. Song, Z. Li, Y. Yang, Y. Wang, X. Wang, and C. Zhu. Gut microbiota changes in patients with autism spectrum disorders. *Journal of psychiatric research*, 129:149–159, 2020. doi: 10.1016/j.jpsychires.2020.06.032.

[115] M. Lou, A. Cao, C. Jin, K. Mi, X. Xiong, Z. Zeng, X. Pan, J. Qie, S. Qiu, Y. Niu, H. Liang, Y. Liu, L. Chen, Z. Liu, Q. Zhao, X. Qiu, Y. Jin, X. Sheng, Z. Hu, G. Jin, others, and Y. Wang. Deviated and early unsustainable stunted development of gut microbiota in children with autism spectrum disorder. *Gut*, 71(8):1588–1599, 2022. doi: 10.1136/gutjnl-2021-325115.

[116] Q. Zhang, R. Zou, M. Guo, M. Duan, Q. Li, and H. Zheng. Comparison of gut microbiota between adults with autism spectrum disorder and obese adults. *Peer-reviewed journal*, 9:e10946, 2021. doi: 10.7717/peerj.10946.

[117] Z. Chen, K. Shi, X. Liu, Y. Dai, Y. Liu, L. Zhang, X. Du, T. Zhu, J. Yu, S. Fang, and F. Li. Gut microbial profile is associated with the severity of social impairment and iq performance in children with autism spectrum disorder. *Frontiers in psychiatry*, 12:789864, 2021. doi: 10.3389/fpsyt.2021.789864.

[118] X. Cao, K. Liu, J. Liu, Y. W. Liu, L. Xu, H. Wang, Y. Zhu, P. Wang, Z. Li, J. Wen, C. Shen, M. Li, Z. Nie, and X. J. Kong. Dysbiotic gut microbiota and dysregulation of cytokine profile in children and teens with autism spectrum disorder. *Frontiers in neuroscience*, 15:635925, 2021. doi: 10.3389/fnins.2021.635925.

[119] S. Ha, D. Oh, S. Lee, J. Park, J. Ahn, S. Choi, and K. A. Cheon. Altered gut microbiota in korean children with autism spectrum disorders. *Nutrients*, 13 (10):3300, 2021. doi: 10.3390/nu13103300.

[120] M. Jendraszak, M. Ga-cka, M. Kotwicka, A. Regdos, M. Pazgrat-Patan, and M. Andrusiewicz. Commercial microbiota test revealed differences in the composition of intestinal microorganisms between children with autism spectrum disorders and neurotypical peers. *Scientific reports*, 11(1):24274, 2021. doi: 10.1038/s41598-021-03794-8.

[121] R. P. Hong, Y. Y. Hou, X. J. Xu, J. D. Lang, Y. F. Jin, X. F. Zeng, X. Zhang, G. Tian, and X. You. The difference of gut microbiota and their correlations with urinary organic acids between autistic children with and without atopic dermatitis. *Frontiers in cellular and infection microbiology*, 12:886196, 2022. doi: 10.3389/fcimb.2022.886196.

[122] O. W. H. Wong, A. M. W. Lam, B. P. N. Or, F. Y. M. Mo, C. K. S. Shea, K. Y. C. Lai, S. L. Ma, S. F. Hung, S. Chan, T. N. Y. Kwong, S. Wong, and P. W. L. Leung. Disentangling the relationship of gut microbiota, functional gastrointestinal disorders and autism: a case-control study on prepubertal chinese boys. *Scientific reports*, 12(1):10659, 2022. doi: 10.1038/s41598-022-14785-8.

[123] Y. C. Chen, H. Y. Lin, Y. Chien, Y. H. Tung, Y. H. Ni, and S. S. Gau. Altered gut microbiota correlates with behavioral problems but not gastrointestinal symptoms in individuals with autism. *Brain, behavior, and immunity*, 106: 161–178, 2022. doi: 10.1016/j.bbi.2022.08.015.

[124] A. B. Haidich. Meta-analysis in medical research. *Hippokratia*, 14(Suppl 1): 29–37, 2010.

[125] C. Andrade. Mean difference, standardized mean difference (smd), and their use in meta-analysis: As simple as it gets. *The Journal of clinical psychiatry*, 81(5):20f, 2020. doi: 10.4088/JCP.20f13681.

[126] P. Schober and T. R. Vetter. Meta-analysis in clinical research. *Anesthesia and analgesia*, 131(4):1090–1091, 2020. doi: 10.1213/ANE.0000000000005001.

[127] Matthew Gurka, George Kelley, and Lloyd Edwards. Fixed and random effects models. *Wiley Interdisciplinary Reviews: Computational Statistics.*, 4, 2012. doi: 10.1002/wics.201.

[128] J. R. Dettori, D. C. Norvell, and J. R. Chapman. Fixed-effect vs random-effects models for meta-analysis: 3 points to consider. *Global spine journal*, 12(7): 1624–1626, 2022. doi: 10.1177/21925682221110527.

[129] L. Lin, H. Chu, and J. S. Hodges. Alternative measures of between-study heterogeneity in meta-analysis: Reducing the impact of outlying studies. *Biometrics*, 73(1):156–166, 2017. doi: 10.1111/biom.12543.

[130] L. Shamseer, D. Moher, M. Clarke, D. Ghersi, A. Liberati, M. Petticrew, P. Shekelle, L. A. Stewart, and Prisma-P Group. Preferred reporting items for systematic review and meta-analysis protocols (prisma-p) 2015: elaboration and explanation. *BMJ: Clinical Research education*, 350:g7647, 2015. doi: 10.1136/bmj.g7647.

[131] T. Engel, S. A. Blowes, D. J. McGlinn, N. J. Gotelli, B. J. McGill, and J. M. Chase. How does variation in total and relative abundance contribute to gradients of species diversity? *Ecology and Evolution*, 12:e9196, 2022.

[132] D. K. Lee, J. In, and S. Lee. Standard deviation and standard error of the mean. *Korean journal of anesthesiology*, 68(3):220–223, 2015. doi: 10.4097/kjae.2015.68.3.220.

[133] A. Hazra. Using the confidence interval confidently. *Journal of thoracic disease*, 9(10):4125–4130, 2017. doi: 10.21037/jtd.2017.09.14.

[134] J. P. Higgins and S. G. Thompson. Quantifying heterogeneity in a meta-analysis. *Statistics in medicine*, 21(11):1539–1558, 2002. doi: 10.1002/sim.1186.

[135] Y. Chang, M. R. Phillips, R. H. Guymer, et al. The 5 min meta-analysis: understanding how to read and interpret a forest plot. *Eye*, 36:673–675, 2022. doi: 10.1038/s41433-021-01867-6.

[136] G. S. Navgire, N. Goel, G. Sawhney, et al. Analysis and interpretation of metagenomics data: an approach. *Biological Procedings Online*, 24:18, 2022. doi: 10.1186/s12575-022-00179-7.

[137] H. B. Hassler, B. Probert, C. Moore, et al. Phylogenies of the 16s rrna gene and its hypervariable regions lack concordance with core genome phylogenies. *Microbiome*, 10:104, 2022. doi: 10.1186/s40168-022-01295-y.

[138] S. Llado Fernandez, T. V-trovsko, and P. Baldrian. The concept of operational taxonomic units revisited: genomes of bacteria that are regarded as closely related are often highly dissimilar. *Folia microbiologica*, 64(1):19–23, 2019. doi: 10.1007/s12223-018-0627-y.

[139] T. V-trovsko and P. Baldrian. The variability of the 16s rrna gene in bacterial genomes and its consequences for bacterial community analyses. *PloS one*, 8 (2):e57923, 2013. doi: 10.1371/journal.pone.0057923.

[140] alvo-i t? M. *Huson, D. H. SILVA, RDP, Greengenes, NCBI and OTT — how do these taxonomies compare?. BMC Genomics*, 18 (Suppl 2), 114, 2017. doi: 10.1186/s12864-017-3501-4.

[141] S. Kochl, H. Niederstotter, and W. Parson. Dna extraction and quantitation of forensic samples using the phenol-chloroform method and real-time pcr. *Methods in molecular biology)*, 297:13–30, 2005. doi: 10.1385/1-59259-867-6:013.

[142] Ashwani Thukral. A review on measurement of alpha diversity in biology. *Agricultural Research Journal.*, 54:1, 2017. doi: 10.5958/2395-146X.2017.00001.1.

[143] M. K. Konopi-ski. Shannon diversity index: a call to replace the original shannon's formula with unbiased estimator in the population genetics studies. *Peer-Reviewed Journal*, 8:e9391, 2020. doi: 10.7717/peerj.9391.

[144] T. Andermann, A. Antonelli, R. L. Barrett, and D. Silvestro. Estimating alpha, beta, and gamma diversity through deep learning. *Frontiers in plant science*, 13:839407, 2022. doi: 10.3389/fpls.2022.839407.

[145] R. G. Wong, J. R. Wu, and G. B. Gloor. Expanding the unifrac toolbox. *PloS one*, 11(9):e0161196, 2016. doi: 10.1371/journal.pone.0161196.

[146] J. Fukuyama, P. J. McMurdie, L. Dethlefsen, D. A. Relman, and S. Holmes. Comparisons of distance methods for combining covariates and abundances in microbiome studies. pacific symposium on biocomputing. *Pacific Symposium on Biocomputing*, pages 213–224, 2012.

[147] C. Lozupone, M. Lladser, D. Knights, et al. Unifrac: an effective distance metric for microbial community comparison. *Multidisciplinary Journal of Microbial Ecology*, 5:169–172, 2011. doi: 10.1038/ismej.2010.133.

[148] Allan Peter Davis, Cynthia J. Grondin, Robin J. Johnson, Daniela Sciaky, Roy McMorran, Jolene Wiegers, Thomas C. Wiegers, and Carolyn J. Mattingly. The comparative toxicogenomics database: update 2019, nucleic acids research. *Issue*, 47:D948–D954, January 2019.

[149] M. Kanehisa, M. Furumichi, M. Tanabe, Y. Sato, and K. Morishima. Kegg: new perspectives on genomes, pathways, diseases and drugs. *Nucleic acids research*, 45(D1):D353–D361, 2017.

[150] Bijay Jassal, Lisa Matthews, Guilherme Viteri, Chuqiao Gong, Pascual Lorente, Antonio Fabregat, Konstantinos Sidiropoulos, Justin Cook, Marc Gillespie, Robin Haw, Fred Loney, Bruce May, Marija Milacic, Karen Rothfels, Cristoffer Sevilla, Veronica Shamovsky, Solomon Shorser, Thawfeek Varusai, Joel Weiser, Guanming Wu, Lincoln Stein, Henning Hermjakob, and Peter D'Eustachio. The reactome pathway knowledgebase, nucleic acids research. *Issue*, 48:D498–D503, January 2020.

[151] L. Coretti, L. Paparo, M. P. Riccio, F. Amato, M. Cuomo, A. Natale, L. Borrelli, G. Corrado, M. Comegna, E. Buommino, G. Castaldo, C. Bravaccio, L. Chiariotti, R. Berni Canani, and F. Lembo. Gut microbiota features in young children with autism spectrum disorders. *Frontiers in microbiology*, 9:3146, 2018. doi: 10.3389/fmicb.2018.03146.

[152] L. N. Dilliraj et al. The evolution of ketosis: Potential impact on clinical conditions. *Nutrients: Open Access Journal of Nutrition*, 14(17):1–17, 2022. doi: 10.3390/nu14173613.

[153] L. Massoni. Folic acid in neuropsychiatric disorders. *Medical Discoveries*, 3(3): 1–11, 2024. doi: 10.52768/2993-1142/1133.

[154] X. Chen, Y. Zhang, H. Wang, L. Liu, W. Li, and P. Xie. The regulatory effects of lactic acid on neuropsychiatric disorders. *Discoveries in Mental Health*, 2(1), 2022. doi: 10.1007/s44192-022-00011-4.

[155] S. Dottorato & T. Biomediche & V. C. Tutor & V. P. Co tutor. Cholesterol metabolism in autism spectrum disorders. pages 1–82, 2023.

[156] R. E. Irwin et al. The interplay between dna methylation, folate and neurocognitive development. *Epigenomics: The Leading International Journal for Epigenetics Research*, 8(6):863–879, 2016. doi: 10.2217/epi-2016-0003.

[157] K. Atarashi, T. Tanoue, M. Ando, N. Kamada, Y. Nagano, S. Narushima, W. Suda, A. Imaoka, H. Setoyama, T. Nagamori, E. Ishikawa, T. Shima, T. Hara, S. Kado, T. Jinnohara, H. Ohno, T. Kondo, K. Toyooka, E. Watanabe, S. Yokoyama, others, and K. Honda. Th17 cell induction by adhesion of microbes to intestinal epithelial cells. *Cell*, 163(2):367–380, 2015. doi: 10.1016/j.cell.2015.08.058.

[158] A. M. Valdes, J. Walter, E. Segal, and T. D. Spector. Role of the gut microbiota in nutrition and health. *BMJ: Clinical Research education*, 2018. doi: 10.1136/bmj.k2179.

[159] G. Rizzatti, L. R. Lopetuso, G. Gibiino, C. Binda, and A. Gasbarrini. Proteobacteria: A common factor in human diseases. *BioMed research international*, 2017, 2017. doi: 10.1155/2017/9351507. Article 9351507.

[160] F. A. Rainey and V. Family. Lachnospiraceae fam. In In Bergey's, editor, *nov*, pages 921–968. Berlin/Heidelberg, Germany, Manual of Systematic Bacteriology, 3rd ed.; De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K. H., Whitman, W. B., Eds.; Springer, 2009.

[161] E. Stackebrandt. The family lachnospiraceae. In E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson, editors, *The Prokaryotes. , Berlin.* Springer, Heidelberg, 2014. doi: 10.1007/978-3-642-30120-9_363.

[162] B. T. Layden, A. R. Angueira, M. Brodsky, V. Durai, and W. L. Lowe. Jr (2013). short chain fatty acids and their receptors: new metabolic targets. *Translational research : the journal of laboratory and clinical medicine*, 161(3):131–140. doi: 10.1016/j.trsl.2012.10.007.

[163] K. M. Maslowski, A. T. Vieira, A. Ng, J. Kranich, F. Sierro, D. Yu, H. C. Schilter, M. S. Rolph, F. Mackay, D. Artis, R. J. Xavier, M. M. Teixeira, and C. R. Mackay. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor gpr43. *Nature*, 461(7268):1282–1286, 2009. doi: 10.1038/nature08530.

[164] L. K. Brahe, A. Astrup, and L. H. Larsen. Is butyrate the link between diet, intestinal microbiota and obesity-related metabolic diseases? *Obesity reviews : an official journal of the International Association for the Study of Obesity*, 14 (12):950–959, 2013. doi: 10.1111/obr.12068.

[165] K. J. Portune, A. Benitez-Paez, E. M. Del Pulgar, V. Cerrudo, and Y. Sanz. Gut microbiota, diet, and obesity-related disorders-the good, the bad, and the future challenges. *Molecular nutrition and food research*, 61(1):10, 2017. doi: 10.1002/mnfr.201600252.

[166] M. X. Byndloss, E. E. Olsan, F. Rivera-Chavez, et al. Microbiota-activated ppar-γ signaling inhibits dysbiotic enterobacteriaceae expansion. *Science*, 357: 570–5, 2017. doi: 10.1126/science.aam9949.

[167] L. Peng, Z.-R. Li, R. S. Green, I. R. Holzman, and J. Lin. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of

amp-activated protein kinase in caco-2 cell monolayers. *Journal of Nutrition.*, 139:1619–1625, 2009.

[168] C. L. Sherry et al. Sickness behavior induced by endotoxin can be mitigated by the dietary soluble fiber, pectin, through upregulation of il-4 and th2 polarization. *Brain. Behavior. Immunity.*, 24:631–640, 2010.