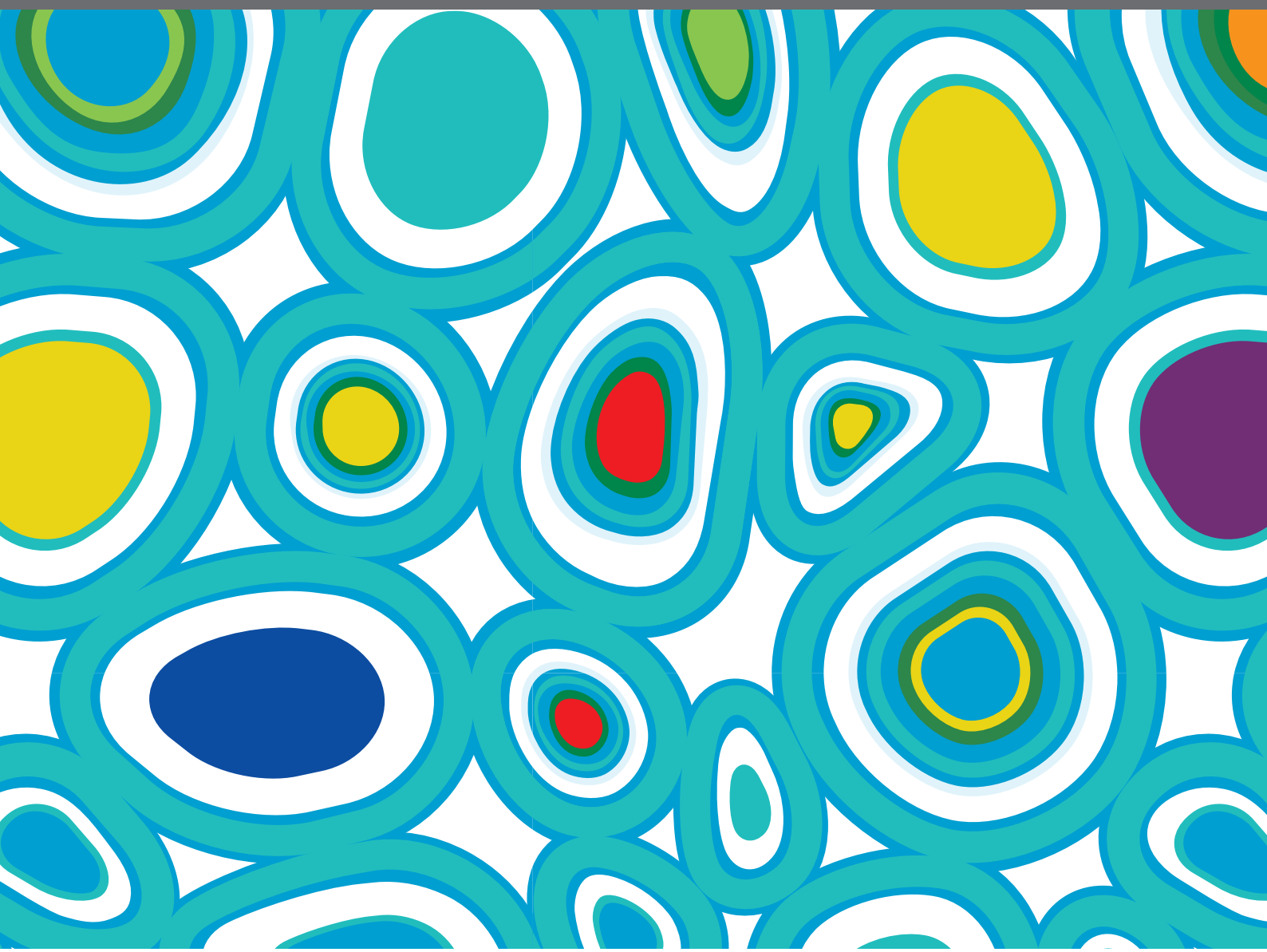
THE GUT MICROBIOTA ORCHESTRATES THE NEURONAL-IMMUNE SYSTEM

EDITED BY: Paola Brun, Hamid I. Akbarali and Ignazio Castagliuolo
PUBLISHED IN: Frontiers in Cell and Developmental Biology and
Frontiers in Cellular and Infection Microbiology



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ISSN 1664-8714
ISBN 978-2-88966-818-2
DOI 10.3389/978-2-88966-818-2

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THE GUT MICROBIOTA ORCHESTRATES THE NEURONAL-IMMUNE SYSTEM

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Citation: Brun, P., Akbarali, H. I., Castagliuolo, I., eds. (2021). The Gut Microbiota Orchestrates The Neuronal-Immune System. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88966-818-2

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frontiers in Cell and Developmental Biology

EDITORIAL
published: 29 March 2021
doi: 10.3389/fcell.2021.672685

Editorial: The Gut Microbiota Orchestrates the Neuronal-Immune System

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Keywords: gut microbiota, inflammation, gut dysmotility, dysbiosis, neuroimmune cross-talk

Editorial on the Research Topic

The Gut Microbiota Orchestrates the Neuronal-Immune System

The Research Topic "The Gut Microbiota Orchestrates The Neuronal-Immune System" was proposed to bring together studies addressing the role of intestinal microbes in determining the homeostasis or the disease both at local and systemic levels. The gut microbiota is the richest and most complex microbial ecosystem in the human body as it is made up of trillions of cells, including bacteria and fungi, whose collective genetic material significantly impacts on host's functions. Moreover, in the gut, unlike in other body compartments, the microbial community is in close contact with enterocytes, enteric neurons, and immune cells. Consolidate knowledge has clarified that alterations in gut microbiota, namely dysbiosis, influence intestinal motility, mucosal permeability, and antigen tolerance. The direct interaction between neuronal and immune cells has been extensively investigated in the last few years, establishing the "neuro-immune cell unit" as the local checkpoint in controlling intestinal functions. However, studies merging the multifaceted role of the gut microbiota in the neuro-immune units are still limited.

In the gut wall, immune cells face food antigens, symbiotic and potentially pathogenic microbes and set a tolerance threshold to prevent aberrant inflammation. Loss of tolerance results in abnormal activation of the immune response and is involved in local disorders ranging from inflammatory bowel diseases to irritable bowel syndrome. In this scenario, the central nervous system interacts with the intestinal immune system to modulate inflammation through humoral and neural pathways, using a mechanism referred to as the intestinal cholinergic anti-inflammatory pathway. On the other side, gut microbes drive the secretion of neuroactive factors to set the enteric neuronal network's activation and function. Thus, the gut microbiota is on the fringes of the neuronal-immune system and gut dysbiosis has dramatic consequences for tissue homeostasis, resulting in intestinal inflammatory diseases and dysmotility.

We are grateful to the 54 Authors from three continents that contributed to this Research Topic with review and original research. Their publications consider the key events orchestrating the impact of the gut microbiota in intestinal and systemic disorders, looking at the fundamental mechanisms and pathways behind the mere alterations in microbiota composition. The main results are outlined in **Figure 1**. The contributions to this Research Topic also clarify the impact of host genetic factors and soluble neurotransmitters in the microbiota-gut-brain axis, paving the way for potential modulators in inflammatory conditions and disorders of the central nervous system. This collection of publications widens knowledge in the field and provides novel insights in supporting experimental models worth considering in future research. In the following, we give an overview of the research articles included in the Research Topic.

Edited and reviewed by:
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Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *26 February 2021*
Accepted: *01 March 2021*
Published: *29 March 2021*

Citation:
Brun P, Akbarali HI and Castagliuolo I (2021) Editorial: The Gut Microbiota Orchestrates the Neuronal-Immune System. Front. Cell Dev. Biol. 9:672685. doi: 10.3389/fcell.2021.672685

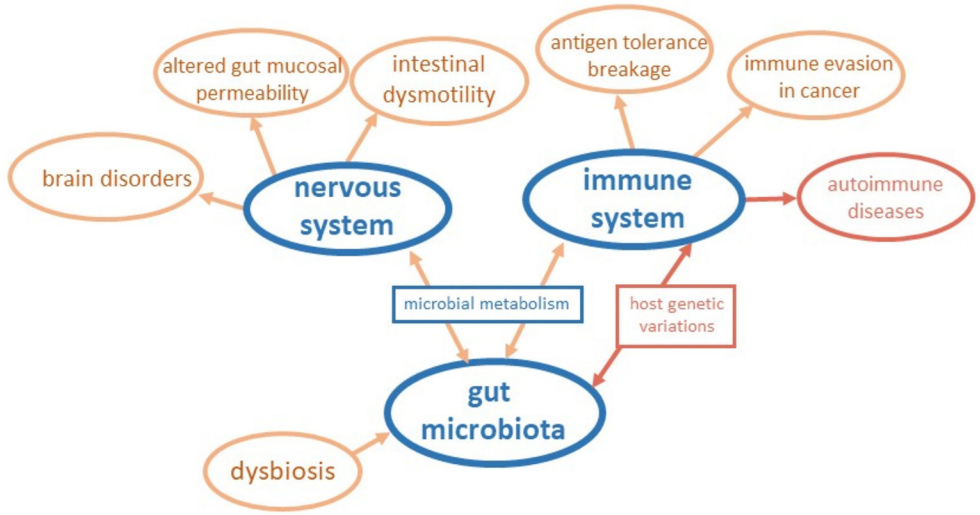

FIGURE 1 | Schematic representation of insights coming from contributions to the Research Topic. The connections among the gut microbiota, the nervous system, and the immune system are outlined considering the main results of papers of the Research Topic.

Dehhaghi et al. reviewed the gut-brain bidirectional pathway in neuroinflammation and brain tumors. They provided state-of-the-art information supporting the involvement of gut microbiota in brain cancers via (i) the kynurenine metabolism, (ii) deprivation of amino acids due to the metabolism of the gut microbiota, and (iii) the microglia dysfunction mediated by immune cells. In the gut, bacteria can reduce the systemic availability of tryptophan and arginine, utilizing them to produce microbial proteins or directing them into microbial metabolic pathways. In humans, the kynurenine pathway is the main route of tryptophan metabolism, and dysregulation of the kynurenine pathway has been linked with cancer development because of the disruption of the antitumoral immune responses. By drawing the complexity of the gut microbiota-brain cancer axis, this review deepens our understanding of microbial metabolism's role in shaping the anti-tumor immune responses, inspiring the development of novel therapeutic strategies.

Metabolites generated in the gut by dysbiotic microbiota modulate brain function with different outcomes and have been correlated with brain disorders. Patients who have schizophrenia have dysregulated microbial profiles. However, clinical data lack consistency and animal models of schizophrenia did not recapitulate gastrointestinal dysfunction or dysbiosis, making the research difficult. Gubert et al. performed gut microbiome profiling in metabotropic glutamate receptor 5 knockout (mGlu5KO) mice, a preclinical model of schizophrenia. By 16S rRNA sequencing of bacterial genomic DNA from fecal samples, they found a significant genotype difference in microbial beta diversity with decreased relative abundance in the *Erysipelotrichaceae* family and *Allobaculum* genus. The differential community composition substantiates the utility of the mGlu5KO animal model for the investigations of gut dysbiosis and related gut microbiota-brain axis signaling as a potential modulator in schizophrenia. Indeed, bacteria in the gut secrete soluble factors that positively influence the psychological status of the host. In their research paper, Beck et al. identified one *Lactobacillus* strain isolated from newborns as psychobiotic. Indeed, the strain has an anti-inflammatory effect (increases production of IL-10), modulates gut microbiota composition (enhances the relative presence of *Bacteroidetes*), and contributes to the gut-brain axis by augmenting serum dopamine level in healthy mice.

Increasing evidence suggests a role for the gut microbiome in autoimmune diseases, including multiple sclerosis, but the impact of host genetic variation in shaping the gut microbiota has been largely overlooked. Kishikawa et al. performed a phylogenetic, functional gene, and pathway analysis of the gut microbiome of Japanese patients with relapsing-remitting multiple sclerosis revealing novel interactions among the gut metagenome and the host genome. In particular, they identified discrepancies in the case-control comparison for gene clades related to immune system function and molecular pathways involved in recognizing lipopolysaccharide. As a result, patients with multiple sclerosis have prompt secretion of inflammatory cytokines during disease relapses, thus highlighting the role of the gut microbiome in the onset and long-term evolution of multiple sclerosis. Alteration in immune response and aberrant recognition of microbial antigens drive functional gastrointestinal disorders such as irritable bowel syndrome (IBS). Arredondo-Hernandez et al. analyzed host single nucleotides polymorphisms (SNPs) and taxonomic composition of the gut microbiota collected from IBS patients and healthy controls selected among Mestizo Latin America, a population suffering from several inflammatory

disorders. They identified 76 SNPs and a relative decrease in *Bacteroidetes* in IBS patients that may serve as a hallmark in IBS diagnosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 25 July 2019
doi: 10.3389/fcimb.2019.00269

Multidisciplinary and Comparative Investigations of Potential Psychobiotic Effects of *Lactobacillus* Strains Isolated From Newborns and Their Impact on Gut Microbiota and Ileal Transcriptome in a Healthy Murine Model

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Psychobiotics are probiotic microorganisms that may exert positive influence on the psychological status of the host. Studies have revealed immunological and microbiological correlations of gut microbiota and the gut-brain axis, and have investigated psychobiotics based on the findings of the gut-brain axis. Considering their mode of actions, the present study sets anti-inflammatory effect, neurotransmitter modulation, and gut microbiota modulation as three essential criteria to evaluate *Lactobacillus casei* ATG-F1 (F1), *L. reuteri* ATG-F3 (F3), and *L. reuteri* ATG-F4 (F4) isolated from newborns as psychobiotics candidates in a healthy mouse model and compares the results with a non-treated control group and an ampicillin-induced gut dysbiosis (Amp) group as a negative control. The F3 and F4 strains showed anti-inflammatory effects *in vitro* in RAW264.7 murine macrophages, and the level of anti-inflammatory cytokine interleukin (IL)-10 increased in ileums of mice orally administered with the F4 strain. Serum dopamine level significantly increased only in the F4-treated group as compared with the control group. Serum serotonin level was unaffected in *Lactobacillus*-treated groups, while a significant decrease in serum serotonin level was observed in the Amp group. Bacteroidetes population increased in fecal samples of the F4-treated group as compared with the control, and *Bacteroidales* S24-7 and *Prevotellaceae* population significantly increased at family level in fecal samples from the F4-treated group as compared with the control. In contrast, the Amp group showed an increase in the level of Proteobacteria and a decrease in the level of Bacteroidetes as compared with the control group. Transcriptome analysis revealed a distinctive clustering in ileums from the F4-treated group as compared to other experimental groups. In addition, the circadian rhythm pathway showed maximum enrichment in ileums of *Lactobacillus*-treated mice, and the F4-treated group showed the highest fold changes in circadian rhythm-related genes (*Dbp*, *Per1*, *Per2*, and *Per3*). Conclusively, *L. reuteri*

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *16 April 2019*
Accepted: *12 July 2019*
Published: *25 July 2019*

Citation:
Beck BR, Park G-S, Jeong DY, Lee YH, Im S, Song WH and Kang J (2019) Multidisciplinary and Comparative Investigations of Potential Psychobiotic Effects of Lactobacillus Strains Isolated From Newborns and Their Impact on Gut Microbiota and Ileal Transcriptome in a Healthy Murine Model. Front. Cell. Infect. Microbiol. 9:269. doi: 10.3389/fcimb.2019.00269

ATG-F4 is suggested as a potential psychobiotics through demonstrations of anti-inflammatory effects, serum dopamine modulation, and gut microbiota modulation in a healthy murine model in the present study. Moreover, we carefully suggest gut circadian rhythm modulation as another important criterion of psychobiotics, which may have an important role in the gut-brain axis.

Keywords: psychobiotics, *Lactobacillus reuteri*, anti-inflammation, neurotransmitters, circadian rhythm, gut microbiota, transcriptomics, metagenomics

INTRODUCTION

Mens sana in corpore sano, a Latin phrase that means a healthy mind in a healthy body, implies that the physical and psychological systems are closely linked together. In other words, the health status of the body influences the mental status. Recent studies on the microbiota-gut-brain axis revealed the interaction, correlation, and association of the gut microbiota with the mental status of the host (Lyte and Cryan, 2014; Johnson and Foster, 2018). Considering the gastrointestinal tract, the gut health status including the gut microbiota may alter the host's mind, as the gut microbiota is described as the third organ (O'hara and Shanahan, 2006; Guinane and Cotter, 2013). Among the members of the native and acquired inhabitants of the gut microbiota, certain psychotropic bacteria are defined as psychobiotics by Dinan et al. these are the probiotics that influence and benefit the mental health of the host (Dinan et al., 2013).

The impact of gut microbiota on the gut-brain axis has been actively studied in microbiology. The association of gut microbiota with unstable mental health or disorders such as anxiety, depression (Foster and Neufeld, 2013), stress susceptibility (De Palma et al., 2014), autism spectrum disorder (Mayer et al., 2014; Li and Zhou, 2016; Kelly et al., 2017), schizophrenia (Dinan et al., 2014; Nemani et al., 2015; Kelly et al., 2017), Parkinson's disease (Mulak and Bonaz, 2015; Houser and Tansey, 2017), and Alzheimer's disease (Bhattacharjee and Lukiw, 2013; Pistollato et al., 2016; Jiang et al., 2017) is widely discussed in the past decade. The gut-brain axis was thought to be bidirectional, from the gut to the brain and from the brain to the gut (Carabotti et al., 2015). Thus, the gut microbiota-gut-brain axis is a very intriguing topic in the investigation of the host-microbiota interaction. One of goals of the researchers in this field is to improve the mental health status of the host through the modulation of microbiota or certain microbial supplementation that gives rise to the concept of psychobiotics.

To understand the mechanisms underlying the action of psychobiotics, several studies have suggested the various modes of actions of the gut-brain axis from immunological, microbiological, and/or psychological perspectives. The interaction between gut microbiota and gut-brain axis may involve immunological mode of actions such as the balance between proinflammatory and anti-inflammatory responses, modulation of cytokine production in the gut, and crosstalk of the immune cells such as dendritic cells, macrophages, and T and B cells responding to commensal or allogenic microorganisms in the gut (Fung et al., 2017; Bambury et al., 2018). Microbial community factors influence the gut-brain axis through Bacteroidetes present in the gut microbiota, Bacteroidetes/Firmicutes ratio, or changes in Proteobacteria population (Dinan and Cryan, 2015; Fung et al., 2017; Kelly et al., 2017). In addition, microbial metabolites such as short-chain fatty acids produced from microbial fermentation (Bambury et al., 2018; Ho et al., 2018) and direct production of neurotransmitters (e.g., dopamine, serotonin, and γ-amino butyric acid) by microorganisms in the gut may contribute to the gut-brain axis modulation (Cryan and Dinan, 2012; Johnson and Foster, 2018).

Based on the findings and hypotheses of previous studies on the gut-brain axis, herein we set three criteria of potential psychobiotic properties, including anti-inflammatory potentials, influence on neurotransmitters, and modulation of gut microbiota, to develop a procedure to evaluate psychobiotics. By focusing on these criteria, we isolated three *Lactobacillus* species from new born infants, namely, *L. casei* ATG-F1 (F1), *L. retueri* ATG-F3 (F3), and *L. reuteri* ATG-F4 (F4), and investigated their potential psychobiotic properties.

METHODS AND MATERIALS

Bioethics Declaration

Ethics approval for animal study was provided by the Institutional Animal Care and Use Committee (IACUC) of AtoGen Co., Ltd., registration number AEC-20181102-0001 from the Animal and Plant Quarantine Agency of South Korea, approval number ATG-IACUC-REV-180810. Animal care and ethics were conducted as per the guidelines of Animal and Plant Quarantine Agency and Ministry of Food and Drug Safety of South Korea.

Animals and Experimental Groups

Four-week-old C57BL/6J male mice were purchased from Central Lab Animal Inc., Seoul. Male mice used to avoid any effects of oestrous cycle and related hormonal changes of female mice. Mice were acclimatized for a week in the laboratory and maintained in a controlled environment with a temperature of 23 ± 0.8°C, humidity of 55 ± 3%, and 12 h day/night cycle. Five mice were placed in Sealsafe NEXT individual ventilation cages (Tecniplast, Italy) and fed *ad libitum* with normal diet for rodents (Cargill Inc., Purina®, Korea). After acclimatization, the control mice received distilled water (DW), while the mice from the Amp group received DW containing 1 g/L ampicillin to induce gut microbiota dysbiosis (Le Bastard et al., 2018). Mice from the F1, F3, and F4 groups received DW containing ∼1.0×10^7 CFU/mL of each *Lactobacillus* strain as drinking

water for 4 weeks. Briefly, *Lactobacillus* strains were overnight cultured in MRS broth for overnight at 37°C, and bacterial cell pellets were harvested by centrifugation at 12,000 ×*g*. Resulting bacterial cell pellets were washed 3 times with DW and suspended to drinking water of each *Lactobacillus* treatment group. Resulting acidity of lactobacilli suspended DW was ∼ pH 7.0. Initial concentrations of lactobacilli were maintained close to the initial concentration at maximum of 3 days at 23 ± 0.8°C (**Figure S1**), and the drinking water containing each *Lactobacillus* strain was changed to new sets of drinking water in 3 days intervals, respectively. Weight, food consumption amount, and water consumption amount were monitored throughout the experiments. At experimental endpoint, fresh fecal samples were collected by placing each mouse in an empty cage for 30 to 45 min. Fecal samples of mice were collected at BSL-2 level with an aid of AIRSTREAM® class II biohazard safety cabinet (ESCO Micro Pte Ltd. Singapore). Serum and ileum samples of mice were collected after anesthetization by diethyl ether. Blood samples for serum collection were collected by heart puncture from each mouse, and freshly obtained serum samples were immediately processed for neurotransmitter ELISA assays to avoid oxidation of neurotransmitters. Mice were sacrificed at 3 h mark of the day cycle. Induction of gut dysbiosis owing to ampicillin treatment was confirmed by the enlargement of cecum of mice from the Amp group (**Figure S2**). Total of two independent sets of animal experiments were performed (total $n = 10$ per experimental group).

Lactobacillus Strains

Two anonymous donors of Daejeon, South Korea, kindly provided fecal samples of newborn babies. *L. casei* ATG-F1 was isolated in January 2016 from the first donor, and *L. reuteri* ATG-F3 and *L. reuteri* ATG-F4 were isolated in June 2018 from the second donor respectively. Fecal samples were opened and processed in BSL-2 AIRSTREAM® class II biohazard safety cabinet for isolation of *Lactobacillus* strains. Each *Lactobacillus* strain was identified through 16S rRNA sequencing. *L. casei* ATG-F1 was used for interspecies comparison and *L. reuteri* strains, for intraspecies comparison. *Lactobacillus* strains were cultured on de Man Rogosa Sharpe (MRS) medium (Difco Laboratories, USA) at 37°C.

Anti-inflammatory Effects of *Lactobacillus* Strains

In vitro Anti-inflammatory Effects of Lactobacillus Strains in Murine Macrophage Model

Murine macrophage RAW264.7 cells were cultured up to ∼80% confluency in complete Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic cocktail (penicillin/streptomycin, Sigma-Aldrich, Germany) at 37°C in 5% CO_2. The cells were collected and seeded at a density of 1×10^6 cells/well in a 24-well cell culture plate (SPL Life Science, Korea) for 24 h. Prior to treatment, *Lactobacillus* strains were lysed by treating the bacterial pellets with lysozyme and sonication, and the lysates were heated at 80°C for 30 min to avoid bacterial growth. About 1 μg/mL of LPS (Sigma-Aldrich, Germany), 100 μg/mL of bacterial lysate, or the combination of LPS and lysate was used treated to cells. Culture supernatants were collected after 24 h of incubation, and IL-10 concentration was determined with IL-10 Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (R&D systems, USA). ELISA results were read with Epoch microplate spectrophotometer (BioTek instruments, USA) at 450 nm wavelength. The inhibition of LPS-induced nitrite production by each lysate was measured by incubating 100 μL of cell culture supernatant and 100 μL of Griess reagent (Sigma-Aldrich, USA) for 10 min at 25°C. The results were analyzed at 540 nm wavelength.

Ileum IL-10 Measurement in Murine Models

Ileum tissue samples weighing 100 mg were homogenized in 400 μL of T-PER™ tissue protein extraction reagent (ThermoFisher Scientific, USA) supplied with cOmplete™ mini EDTA-free protease inhibitor cocktail (Merck, Germany) using a hand-held homogenizer (T10 Basic, IKA®, China). Lysates were centrifuged at 10,000 ×*g* for 20 min, and the resulting supernatants were analyzed with IL-10 Quantikine ELISA Kit (R&D systems, USA) to measure IL-10 concentration.

Serum Dopamine and Serotonin Measurement

For serum dopamine level measurement, 200 μL of serum samples were mixed 100 μL of 1× phosphate-buffered saline (PBS) to adjust the dopamine extraction reaction volume, and subjected to the extraction procedures provided in dopamine ELISA kit (Abnova, Taiwan). For serum serotonin measurement, serum samples were diluted at 1:5 and 1:10 ratios and used with serotonin ELISA kit (Abcam, USA). Competitive ELISA experiments were performed following the manufacturer's instructions.

Fecal Microbiota Analysis

Genomic DNA extraction from fecal samples was performed using the PowerFecal DNA Isolation Kit (Mo Bio Laboratories, USA). The quantity and quality of extracted DNA were measured using NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis, respectively. The V3–V4 hypervariable regions of the bacterial 16S rRNA were amplified with unique 8 bp barcodes and sequenced on the Illumina MiSeq PE300 platform according to standard protocol (Caporaso et al., 2012). The raw sequence data were submitted to the NCBI's SRA database (NCBI BioProject PRJNA516311). Raw reads were analyzed using the QIIME pipeline (Caporaso et al., 2010). Sequences were quality filtered and clustered into operational taxonomic units at 97% sequence identity according to the SILVA 128 database (Yilmaz et al., 2014). The operational taxonomy units were identified at phylum to genus levels. The unweighted and weighted UniFrac distances were previously obtained and used for PCoA (Lozupone et al., 2010).

Transcriptome Analysis

Three randomly selected ileum samples from each experimental group of the first animal experiment set were stabilized and

preserved in RNAlater solution (Invitrogen, USA) and sent to Macrogen Inc., Korea, library preparation (TruSeq RNA Sample Prep Kit v2, Illumina, USA). RNA sequencing (100 bp, NovaSeq 6000 S4 Reagent Kit, Illumina, USA) was performed to produce raw data (Martin and Wang, 2011). The resulting RNA sequencing results were quality checked using FastQC and trimmed (Illumina adapters) through Trimmomatic (Bolger et al., 2014). All sequences were mapped using HISAT2 (Pertea et al., 2016) and analyzed using SAM tools v1.9 to produce a list of significant differentially expressed genes (DEGs, statistical significance cut-off $p < 0.05$) as compared with the control group. Gene ontology (GO) enrichment analysis of significant DEGs of each experimental group compared to the control group was performed using database of Gene Ontology (http://geneontology.org/). Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analysis (Altermann and Klaenhammer, 2005; Kanehisa et al., 2016). Based on the DEG result, the genes of interest were selected and a heatmap was generated using online tool Morpheus (Broad Institute, https://software.broadinstitute.org/morpheus/). The raw sequencing data produced in the present study were deposited in NCBI's SRA database (NCBI BioProject PRJNA52169).

Statistics

One-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was performed to compare multiple columns. Unpaired two-tailed *t*-test was used to separately compare two independent groups. For statistical analysis of transcriptome results, absolute values of transcript fold changes equal or larger than 2.0 were processed with independent *t*-tests. Gene-set enrichment analysis was performed through the modified fisher's exact test to analyze significances of KEGG pathways generated based on transcriptome results.

RESULTS

Anti-inflammatory Effects of *Lactobacillus* Strains

As a mode of action, the anti-inflammatory potentials of *Lactobacillus* strains were evaluated. The concentration of the anti-inflammatory cytokine interleukin (IL)-10 was elevated following co-treatment of RAW264.7 cells with lysates of all the three strains and lipopolysaccharide (LPS) as compared to treatment with LPS only (**Figure 1A**). In comparison with the control group, the F3 and F4 lysates induced higher production of IL-10 by RAW264.7 cells following a single treatment of each lysate without LPS co-treatment ($p < 0.01$, determined by *t*-test). LPS-induced nitrite production by RAW264.7 cell was significantly reduced following treatment with the F3 and F4 lysates, while treatment with the F1 lysate had no effect on nitrite production. The F4 lysate treatment showed the highest reduction in nitrite level among all treatment groups (**Figure 1B**). In animal experiments, IL-10 concentration significantly increased in the ileums of the F4-administrated group as compared with ileums from the control group and the ampicillin-induced gut dysbiosis (Amp) group at the end of 4 weeks of *Lactobacillus* administration (**Figure 1C**). Taken together, only the F4 strain showed anti-inflammatory potentials in both *in vitro* and *in vivo* experiments.

Influence of Lactobacilli on the Levels of Circulatory Dopamine and Serotonin

To examine the effects of lactobacilli on circulatory neurotransmitters, serum dopamine and serotonin levels were measured from the serum samples of each experimental group at the end of 4 weeks from each *Lactobacillus* oral administration. A significant increase in serum dopamine concentration was observed in the F4-treated group as compared with the control and Amp groups. The F1-treated and F3-treated groups showed an increase in the level of serum dopamine as compared with Amp group (**Figure 2A**). However, serum dopamine levels showed variations in the F1 and F3 groups and exhibited no statistical significance as compared with the control group. A minor decrease in serum dopamine level was observed in the Amp group that was not significantly different as compared with control group. Serum serotonin level was unaffected following oral treatment with *Lactobacillus* strains as compared with the control group; however, a significant decrease in serum serotonin level was observed in the Amp group as compared to other experimental groups (**Figure 2B**). These results suggest that the oral microbial supplementation or gut microbiota disruption may influence the levels of circulatory neurotransmitters of the host.

Modulation of Gut Microbiota by Lactobacilli

To examine the effects of lactobacilli on the gut microbiota, the bacterial community from fecal samples of each experimental group was analyzed. The alpha-diversity analysis revealed a significant increase in the population of Bacteroidetes in the F4-treated group as compared to other groups, while the Amp group showed a significant increase in the abundance of Proteobacteria (**Figures 3A,B**, **Figure S3**). The families *Bacteroidales* S24-7 (phylum Bacteroidetes) and *Enterobacteriaceae* (phylum Proteobacteria) showed identical patterns of population shifts in the family level community analysis as compared with phylum level analysis (**Figure 3C**, **Figures S4A–C**). Only the F4-treated group showed a significant increase in the abundance of the family *Prevotellaceae* (phylum Bacteroidetes) as compared with the control or Amp group, while *Lactobacillaceae* population significantly increased only in the F4-treated group as compared to all other experimental groups (**Figures S4B,D**).

Beta-diversity was examined by the principal coordinate analysis (PCoA) to compare the composition of microbiota using UniFrac distance. Weighted-UniFrac takes into account the relative abundance of species/taxa shared between samples, whereas unweighted-UniFrac only considers presence/absence. The microbiota from the Amp group separated from those of other groups (**Figure 4**). The composition of microbiota of the F4-treated group was not distinct from that of control group in the unweighted PCoA plot (**Figure 4A**), whereas the microbial population showed a shift in the F4-treated group, as shown

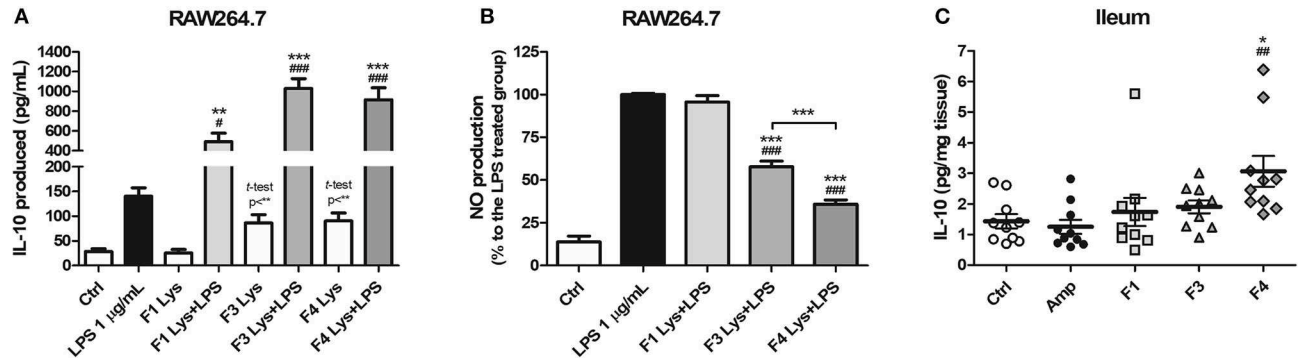

FIGURE 1 | Anti-inflammatory effects of *Lactobacillus casei* ATG-F1 (F1), *L. reuteri* ATG-F3 (F3), and *L. reuteri* ATG-F4 (F4). **(A)** *in vitro* IL-10 production was determined in murine macrophage RAW264.7 cells treated with 100 μg/mL of each *Lactobacillus* lysate with or without LPS co-treatment. **(B)** Nitrite reduction in the LPS co-treated groups. Results of three independent experiments of triplicate wells are shown as mean ± SEM. **(C)** *in vivo* IL-10 concentration in the ileum was determined after the oral administration of lactobacilli and compared with the control (Ctrl) and ampicillin-induced gut dysbiosis (Amp) groups after 4 weeks of the experiment in healthy C57BL/6J mice. Each dot represents one mouse ($n = 10$ per each group). For **(A)** and **(B)**, statistical significance was as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$ vs. the Ctrl group; **(A)** $^{\#}p < 0.05$, $^{\#\#\#}p < 0.0001$ vs. the LPS group; **(B)** $^{***}p < 0.0001$ vs. the LPS group; **(B)** $^{\#\#\#}p < 0.0001$ vs. F1 group. For **(C)**, statistical significance was compared to the Ctrl ($^{*}p < 0.05$) and Amp group ($^{\#\#}p < 0.01$). Statistics were determined with one-way ANOVA along with Tukey's *post-hoc* test for all experiments, and additional *t*-test was used in **(A)** to separately compare the Ctrl group and *Lactobacillus* lysate-treated without LPS treatment groups.

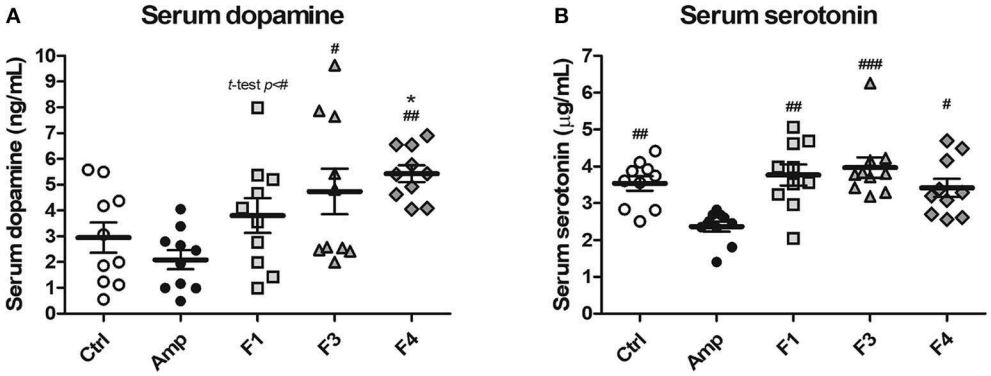

FIGURE 2 | Serum dopamine and serotonin modulation by oral administration of *Lactobacillus* strains for 4 weeks in healthy C57BL/6J mice. Serum **(A)** dopamine and **(B)** serotonin concentration in the groups treated with *Lactobacillus casei* ATG-F1 (F1), *L. reuteri* ATG-F3 (F3), and *L. reuteri* ATG-F4 (F4) as compared to the control group (Ctrl) and ampicillin-induced gut dysbiosis (Amp) group. Each dot represents one mouse ($n = 10$ each group). Statistical significance was as follows: $^{*}p < 0.05$ vs. the Ctrl group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.0001$ vs. the Amp group. Statistics were determined with one-way ANOVA along with Tukey's *post-hoc* test for all experiments, while additional *t*-test was performed in **(A)** to separately compare F1 and Amp groups.

in weighted PCoA plot (**Figure 4B**). Mirroring the α-diversity differences, there were differences in clustering of *Lactobacillus* treated groups compared to the control group and Amp group. However, the change in the microbial population in the F1- and F3-treated groups was inconsistent as compared to the control and F4-treated groups. In the case of the F1- and F3-treated groups, they resulted with different changes between biological replicates, while the F4 treated group showed relatively similar changes between each mouse in the same group. The microbial community scattering shown in the F1- and F3-treated group may be affected by initial gut microbiota of each mouse, however, the F4 treatment seems to force and maintain the abundance of phylum Bacteroidetes at higher level compared to the control group as shown in weighted PCoA plot (**Figure 4B**).

Transcriptional Modulation by Lactobacilli in the Ileum of Mice

Overall transcriptome one-way hierarchical clustering analysis revealed three distinct clusters, namely the control, Amp, and *Lactobacillus*-treated groups (**Figure 5A**). Each sample from the F4-treated group showed the closest grouping in the transcriptome among *Lactobacillus*-treated groups in principal component scatter plot analysis (**Figure 5B**). A total of 142 significant genes with satisfactory cut-off values ($|\text{fold change}| \geq 2$, $p < 0.05$) were detected in the experiment; the number of DEGs from each experimental group as compared with the control group are as follows: the Amp group, 61 genes; the F1-treated group, 37 genes; the F3-treated group, 45 genes; the F4-treated group, 84 genes (**Figure S5**).

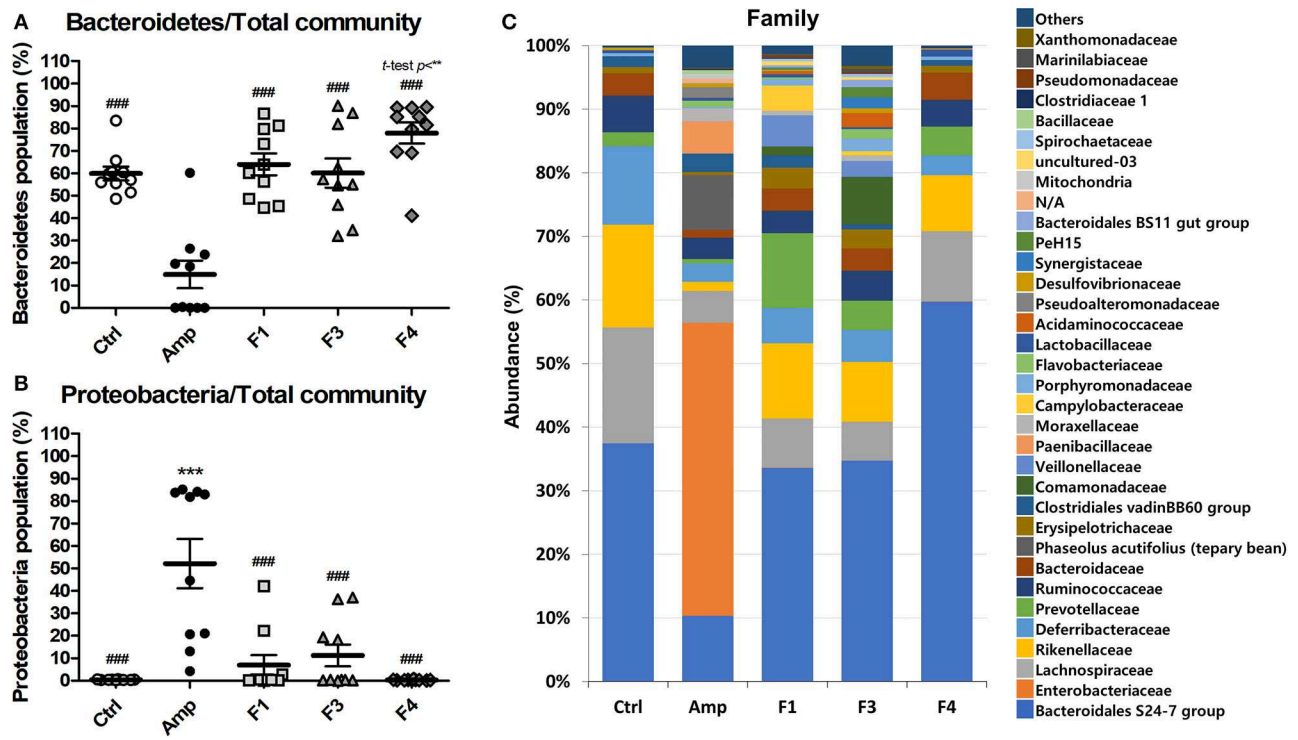

FIGURE 3 | Changes in fecal bacterial community in the ampicillin-induced gut dysbiosis group (Amp) and the groups treated with *Lactobacillus casei* ATG-F1 (F1), *L. reuteri* ATG-F3 (F3), and *L. reuteri* ATG-F4 (F4) as compared to the control (Ctrl) group. Abundance of the phyla **(A)** Bacteroidetes and **(B)** Proteobacteria are shown in dot plot (total read counts). Each dot represents one mouse ($n = 10$ per group). Statistical significance was as follows: $^{**}p < 0.01$, $^{***}p < 0.0001$ vs. the Ctrl group; $\#\#\#p < 0.0001$ vs. the Amp group. Statistics were determined with one-way ANOVA along with Tukey's *post-hoc* test, and *t*-test was performed in **(A)** to compare the F4 and Ctrl groups. Top 35 abundant family level taxa in average of 10 mice from each experimental group were shown in **(C)** stacked bar plot.

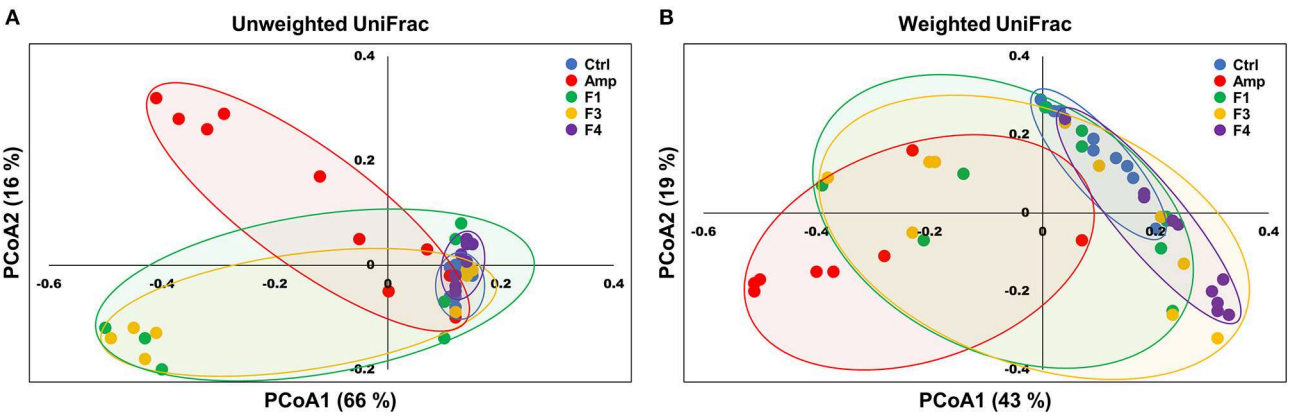

FIGURE 4 | Principal coordinate analysis (PCoA) plots of fecal bacterial communities of the control (Ctrl), ampicillin-induced gut dysbiosis (Amp), *Lactobacillus casei* ATG-F1 (F1)-, *L. reuteri* ATG-F3 (F3)-, and *L. reuteri* ATG-F4 (F4)-treated groups at the end of 4 weeks of oral administration. Plots based on **(A)** unweighted and **(B)** weighted UniFrac distance metrics are shown. Each dot represents one mouse ($n = 10$ per group).

KEGG pathway enrichment was performed using the data set generated from transcriptome analysis of ileum tissue samples. GO enrichment analysis results are provided as **Supplementary Materials** (**Figure S6**, **Tables S1–S4**). Metabolic pathways, circadian rhythm, carbohydrate digestion and absorption, thyroid hormone synthesis, mineral absorption, galactose metabolism, starch and sucrose metabolism, circadian entrainment, steroid hormone biosynthesis, and 5′-AMP-activated protein kinase signaling pathway were identified as the top 10 KEGG enriched pathways in the ileum of

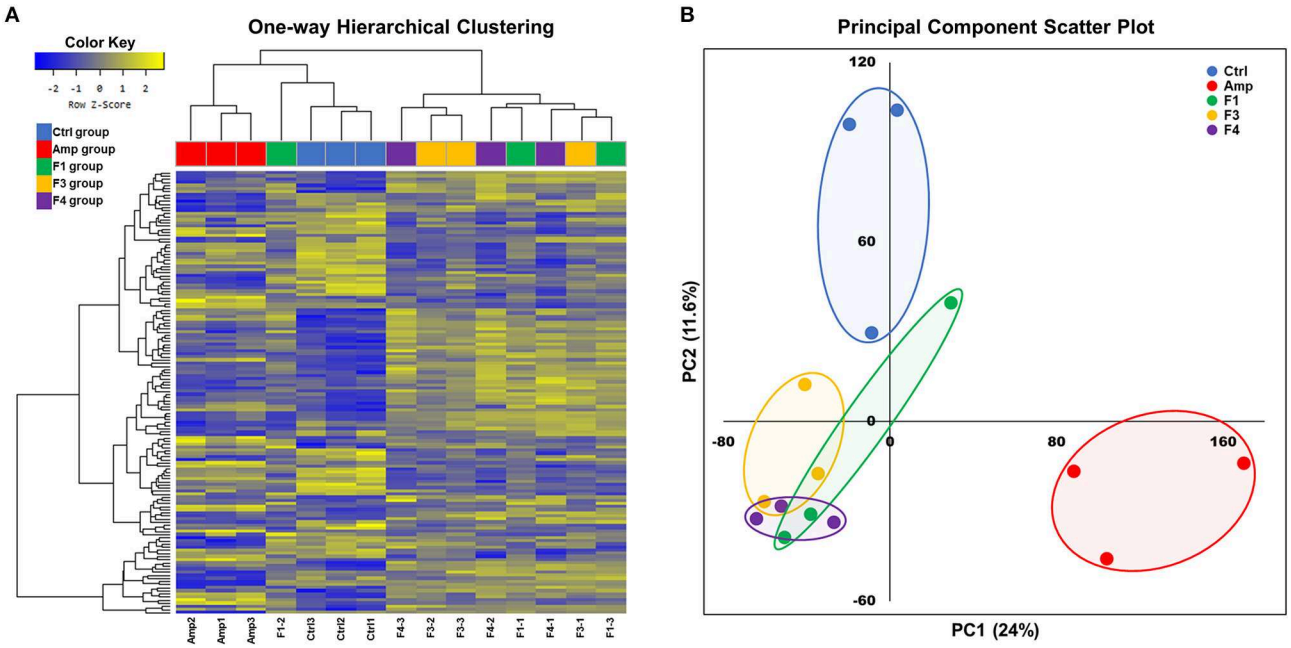

FIGURE 5 | Overall transcriptome analysis results. **(A)** Hierarchical clustering of the gene with significant changes (142 genes, |fold change| ≥2, $p < 0.05$ determined by independent *t*-test), and **(B)** principal component scatter plot of transcriptomes of the ileum samples from each experimental group. Experimental groups ($n = 3$ per group) shown in plots are as follows: Ctrl, the control group; Amp, the ampicillin-induced gut dysbiosis group; F1, *Lactobacillus casei* ATG-F1-treated group; F3, *L. reuteri* ATG-F3-treated group; F4, *L. reuteri* ATG-F4-treated group.

Lactobacillus-treated groups after excluding disease and infection model pathways (**Figure 6**). Transcriptional modulations of circadian rhythm and circadian entrainment were affected following *Lactobacillus* treatments as compared with the control group, while the Amp group showed no significant response in these two categories.

Based on the KEGG pathway enrichment analysis, 15 significantly modulated gene sets that may relate to the alteration of mental status and gut dysbiosis were plotted as a heatmap (**Figure 7A**). Circadian rhythm-related genes (*Dbp*, *Per1*, *Per2*, *Per3*, *Nrld1*, *Nrld2*, *Ciart*, *Cipc*, *Npas2*, and *Arntl*) and steroid hormone biosynthesis-related genes (*Hsd3b2*, *Hsd3b3*, and *Hsd11b2*) were significantly modulated by all *Lactobacillus* strains, and the strongest degree of transcriptional modulation was observed in the F4-treated group. No significant modulation of immune functions was observed among the control and *Lactobacillus*-treated groups, but the Amp group showed a significant decrease in *Jchain* and *C3* genes in transcriptome analysis. Among circadian rhythm-related genes, *Dbp* expression was notably increased in *Lactobacillus*-treated groups as compared to the control and Amp groups, and the F4-treated group showed the highest fold increase as compared to the F1- and F3-treated groups. Correlation was reported in the expression patterns between *Dbp* and circadian rhythm genes (**Figures 7B–G**). Circadian rhythm genes *Per1*, *Per2*, *Per3* (**Figures 7C–E**) showed a negative correlation with *Arntl* and *Npas2* (**Figures 7F,G**), the negatively regulated genes in the circadian rhythm pathway.

DISCUSSION

IL-10, a key cytokine, plays an important role in immune regulation and suppression of inflammation and is produced by dendritic cells, macrophages, and regulatory T cells following pathological inflammatory immune responses (Saraiva and O'garra, 2010; Ouyang et al., 2011). Circulating LPS is one of the important pathogenic factors in neuroinflammation in the host body (Qin et al., 2007). Circulating LPS is derived from gram-negative bacteria from the gut during gut barrier dysfunction, a condition termed as "leaky gut" owing to chronic inflammation (Maes et al., 2008). This phenomenon negatively influences the mind status of the host through the gut-brain axis through neuroinflammation (Wang et al., 2010). The establishment of an immunological status potentiated to anti-inflammation which is described as a regulatory immune tone by Beck et al. (2016) in the gut as a response to probiotics may decrease the permeability of the gut and prevent "leaky gut." Based on contributions of anti-inflammatory effects on neuroprotection, several studies demonstrated neuroprotective effects of lactobacilli or lactobacilli-fermented products (Musa et al., 2017; Yim et al., 2018; Wang et al., 2019). Therefore, the anti-inflammatory potentials of the F4 strain both *in vitro* IL-10 stimulation and inhibition of NO production in murine macrophage cell model and increased IL-10 concentration in the ileums of F4-treated group may suggest the mode of actions of this potential psychobiotic bacterium, as evident from the well-reviewed interactions between the

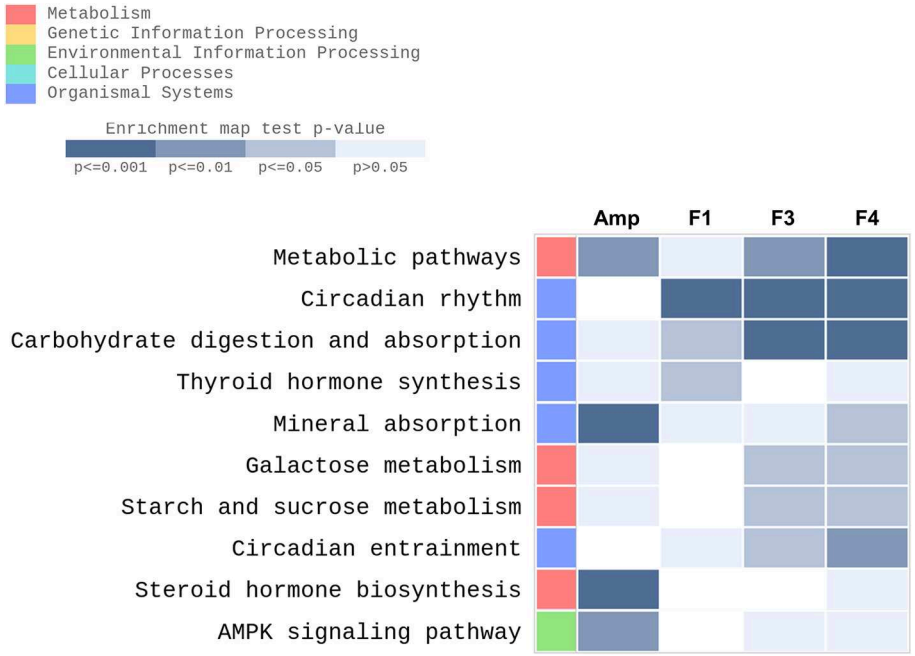

FIGURE 6 | Top 10 KEGG enriched pathways determined in the ileum of experimental groups ($n = 3$ per group) with gene-set enrichment analysis through modified fisher's exact test as compared with the control group were plotted as a heatmap. Experimental groups shown in the heatmap are as follows: Amp, the ampicillin-induced gut dysbiosis group; F1, *Lactobacillus casei* ATG-F1-treated group; F3, *L. reuteri* ATG-F3-treated group; F4, *L. reuteri* ATG-F4-treated group.

immune and nervous system through the gut microbiota by Fung et al. (2017).

As a neurotransmitter and neuromodulator, dopamine plays an important role in tuning the psychological status of the host in several psychosis symptoms (Dobryakova et al., 2015; Ayano, 2016). For instance, a decrease in dopamine level was associated with attention deficit hyperactivity disorder and Parkinson's disease (Ayano, 2016), while any increase in the level of circulatory dopamine, as seen following the F4 treatment, may contribute to the prevention of such psychosis. Consistent with the result of transcriptomics, the expression of the genes related to steroid hormone biosynthesis (*Hsd3b2*, *Hsd3b3*, and *Hsd11b2*) increased following the F4 treatment; Hsd11b2 enzyme (11-β-hydroxysteroid dehydrogenase 2) is involved in the conversion of cortisol to cortisone (Quinkler and Stewart, 2003). The increase in the expression of *Hsd11b2* may contribute to stress tolerance and dopamine overexpression via cortisol reduction, wherein neurotransmitters and cortisol show a negative correlation as demonstrated in other studies (Field et al., 2005; Issa et al., 2010). Although the association between mental safety and observed increment in dopamine level by the F4 strain needs to be confirmed in further studies, no symptoms of possible psychosis, such as anxiety, abnormal behaviors, self-mutilation, or physiological changes (body weight, amount of feed and water consumption), were observed in the present study as a response to increased dopamine level (**Figure S7**). In contrast, the level of serotonin was unaffected by *Lactobacillus* treatment; however, a significant decrease in serum serotonin level was reported in the Amp group, suggesting that gut dysbiosis may have contributed to the development of psychosis because serotonin is an important factor related to mood and cognition (Jenkins et al., 2016). As per the results of the present study, unlike dopamine, serotonin levels were maintained among the experimental groups during continuous *Lactobacillus* administration except in the Amp group. The increase in serotonin level observed in autism spectrum disorder (Gabriele et al., 2014), and this may indirectly support the safety of serum neurotransmitter modulation following the F4 treatment in psychological aspects, as the serum serotonin level was unaffected. However, the present study evaluated serum circulating dopamine and serotonin which limits their influences to the peripheral system, and yet it is not clear where the source of increased dopamine is. Moreover, neurotransmitters cannot pass through the blood-brain barrier, but the interactions in gut-brain axis are reported numerous times. To understand gut and gut microbiota to brain communication, the vagus nerve is suspected and studied as a route of communication between gut-derived or gut microbiota derived-neurotransmitters (Bercik et al., 2011; Bravo et al., 2011; Perez-Burgos et al., 2012; Bonaz et al., 2018). Nevertheless, serum neurotransmitters are possible core markers of psychobiotics.

The supplementation with probiotic strains may change the intestinal microbiome, but the microbial community shifts following probiotic administration seems to be a strain-specific effect. Although the F3 and F4 strains belong to the same species (*L. reuteri*), their influence on the structure of the microbial community was different. The F1 (*L. casei*) and F3 treatment resulted in similar community shifts in the

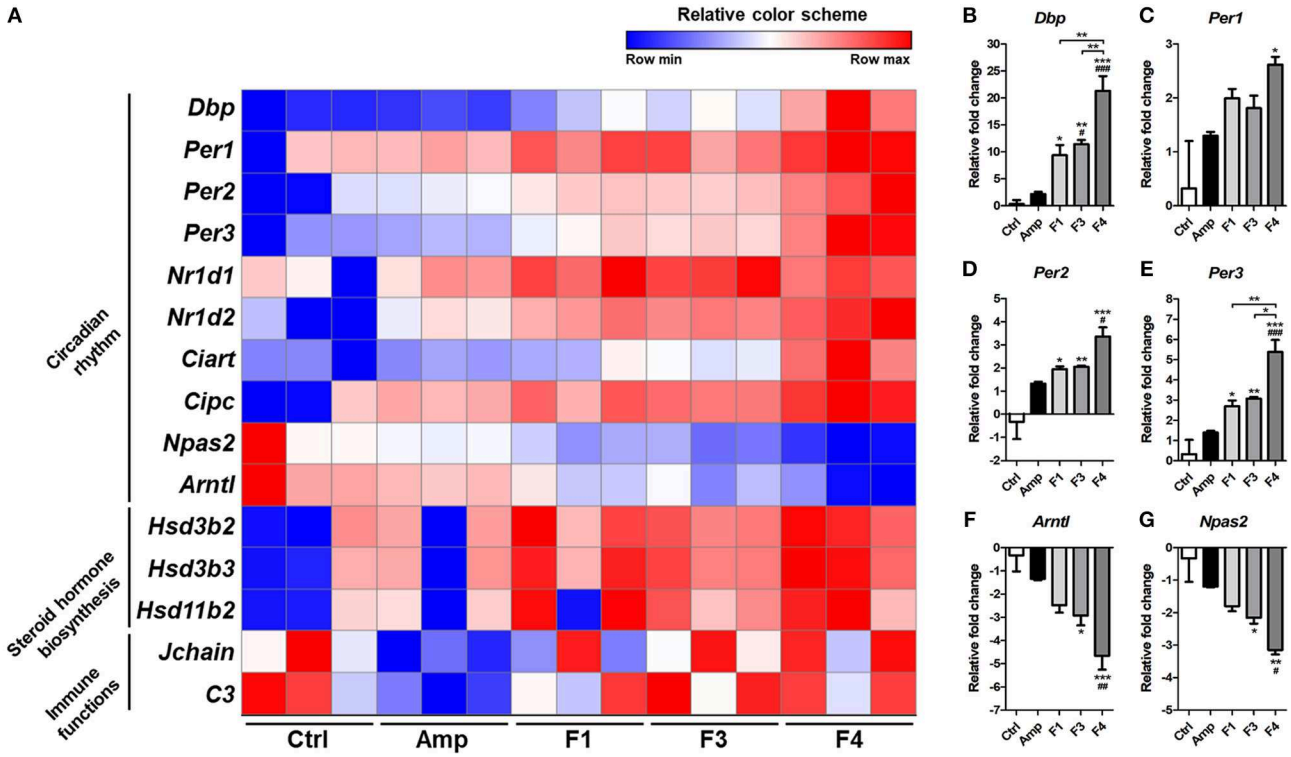

FIGURE 7 | (A) Heatmap of 15 selected genes based on KEGG pathway enrichment shown as a relative color scheme for each row. **(B–G)** Significant relative fold changes in circadian rhythm-associated genes plotted as bar graphs. Experimental groups ($n = 3$ per group) shown in the heatmap and bar graphs are as follows: Ctrl, the control group; Amp, the ampicillin-induced gut dysbiosis group; F1, *Lactobacillus casei* ATG-F1-treated group; F3, *L. reuteri* ATG-F3-treated group; F4, *L. reuteri* ATG-F4-treated group. For the graph of relative fold change, statistical significance was tested with one-way ANOVA along with Tukey's *post-hoc* test as follows: $^{*}p < 0.05$. $^{**}p < 0.01$, $^{***}p < 0.0001$ vs. Ctrl group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.0001$ vs. Amp group.

analysis of microbiota. The F4 treatment induced a significant microbial community shift which resulted in the domination of phylum Bacteroidetes, including *Bacteroidales* S24 group and *Prevotellaceae*, as compared to all other experimental groups. Although modulation of Bacteroidetes population through probiotics administration that links to mental illness or mood disorder is scarce yet, one study reports increased Bacteroidetes population along with mental health improvements after *Bifidobacterium infantis* M-63 intervention in a human trial (Ma et al., 2019). Yet more studies are warranted for the identification of active substances from the F4 strain, it is intriguing that the F4 treatment resulted in a consistent microbial shift in each F4-treated mouse. For example, one study demonstrated that the exopolysaccharides of *L. reuteri* may benefit the growth of *Bacteroides thetaiotaomicron* (Van Bueren et al., 2015). The change in Bacteroidetes population in the gut microbiota correlates with mental health (Dinan and Cryan, 2015; Fung et al., 2017); thus, the F4 strain is a potential psychobiotic bacterium owing to its ability to enhance Bacteroidetes population.

Circadian rhythm or the biological clock that runs at ∼24 h intervals, seems to be another factor that may influence the psychological status of the host (Mcclung, 2013). The circadian rhythm compartments such as *Per*, *Arntl*, or *Npas2* genes are thought to be closely related to mood disorders (Nievergelt et al., 2006; Partonen et al., 2007). In particular, circadian rhythms in the peripheral tissues, or gut in this case, are associated with the immunity, metabolism, and barrier function in the gut (Konturek et al., 2011). Both the host factors as well as the gut microbiota interact with the circadian rhythm of the host (Voigt et al., 2014; Rosselot et al., 2016; Thaiss et al., 2016). *Dbp* is suspected to be related to the circadian oscillation (Stratmann et al., 2010) and its expression was notably increased following *Lactobacillus* treatments; the highest expression level was reported in the F4-treated group. In addition, the strong influence of F4 strain on the circadian rhythm was observed through transcriptomic and KEGG pathway analyses through the upregulation of genes such as *Per1*, *Per2*, and *Per3* along with *Dbp* (**Figure S8**). A similar increase in the oscillation of circadian rhythm was reported in a dietary restriction *Drosophila* model study related to increased fat metabolism and life span (Katewa et al., 2016). The present study showed significant enrichment of the metabolic pathway category in KEGG pathway analysis following treatment with the F4 strain. The dysfunction in the circadian rhythm was thought as a risk factor in Parkinson's disease (Lauretti et al., 2017), and recent reports have confirmed the close association between circadian rhythm as well as dopamine and human mood (Radwan et al., 2018). These findings need further validation to reveal the link with the mechanism of psychobiotics in future. Thus, the present

study carefully suggests that the circadian rhythm function may be one of important markers and criteria of psychobiotics.

To conclude, the observations such as increased anti-inflammatory potential, upregulated serum dopamine expression, and enhanced *Bacteroidetes* population in *L. reuteri* ATG-F4-treated mice demonstrate the psychobiotic potentials of *L. reuteri* ATG-F4, which showed the highest modulation of intestinal circadian rhythm compartments. The present study also suggests that the psychobiotic effects are species- and strain-specific. Although psychobiotic candidates must be investigated for their effects on various mental illness models and further explored to facilitate the development of effective psychobiotics, we carefully suggest the criteria examined in the present study, including the combination of anti-inflammatory effect, influence on host neurotransmitters, gut microbiota modulation, and circadian rhythm modulation, as effective markers for the screening of the potential psychobiotics to improve mental health.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available at NCBI's repository. The raw sequence data of bacterial community sequencing are submitted to NCBI SRA database (NCBI BioProject PRJNA516311, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA516311). The sequencing raw data of transcriptome analysis discussed in this publication are deposited at NCBI SRA database (NCBI BioProject PRJNA52169, https://www.ncbi.nlm.nih.gov/bioproject/521649).

ETHICS STATEMENT

Ethics approval for animal study was provided by the Institutional Animal Care and Use Committee (IACUC) of AtoGen Co., Ltd., registration number AEC-20181102-0001 from the Animal and Plant Quarantine Agency of South Korea, approval number ATG-IACUCREV-180810. Animal care and ethics were conducted as per the guidelines of Animal and Plant Quarantine Agency and Ministry of Food and Drug Safety of South Korea.

AUTHOR CONTRIBUTIONS

BB conceived and designed experiments, isolated strains F3 and F4, conducted animal experiments, and participated in writing, editing, and the correspondence of the manuscript. G-SP produced and analyzed meta-analyses data and participated in manuscript writing. DJ and YL participated in *in vitro* and *in vivo* assays with cells and animals. SI and WS were involved in the characterization, cultivation, and management of bacterial strains. JK isolated and provided strain F1 and were involved in manuscript editing and correspondence.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00269/full#supplementary-material

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Conflict of Interest Statement: All authors were employed by AtoGen Co., Ltd. The authors declare that this study received no third-party funding, and AtoGen Co., Ltd. supported the present study with non-financial supports.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 20 March 2020
doi: 10.3389/fcimb.2020.00072

Mucosal Microbiome Profiles Polygenic Irritable Bowel Syndrome in Mestizo Individuals

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Irritable bowel syndrome (IBS) is the most frequent functional gastrointestinal disorder, worldwide, with a high prevalence among Mestizo Latin Americans. Because several inflammatory disorders appear to affect this population, a further understanding of host genomic background variants, in conjunction with colonic mucosa dysbiosis, is necessary to determine IBS physiopathology and the effects of environmental pressures. Using a simple polygenic model, host single nucleotide polymorphisms (SNPs) and the taxonomic compositions of microbiota were compared between IBS patients and healthy subjects. As proof of concept, five IBS-Rome III patients and five healthy controls (HCs) were systematically studied. The human and bacterial intestinal metagenome of each subject was taxonomically annotated and screened for previously annotated IBS, ulcerative colitis, and Crohn's disease-associated SNPs or taxon abundance. Dietary data and fecal markers were collected and associated with the intestinal microbiome. However, more than 1,000 variants were found, and at least 76 SNPs differentiated IBS patients from HCs, as did associations with 4 phyla and 10 bacterial genera. In this study, we found elements supporting a polygenic background, with frequent variants, among the Mestizo population, and the colonic mucosal enrichment of *Bacteroides*, *Alteromonas*, *Neisseria*, *Streptococcus*, and *Microbacterium*, may serve as a hallmark for IBS.

Keywords: IBS, SNPs, microbiota, *Bacteroides*, polygenic SNPs

OPEN ACCESS

Edited by:
Paola Brun,
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Reviewed by:
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Wioletta Izabela Wujcicka,
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Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *16 October 2019*
Accepted: *13 February 2020*
Published: *20 March 2020*

Citation:
Arredondo-Hernández R, Schmulson M, Orduña P, López-Leal G, Zarate A-M, Alanis-Funes G, Alcaraz LD, Santiago-Cruz R, Cevallos MA, Villa AR, Ponce-de-León Rosales S and López-Vidal Y (2020) Mucosal Microbiome Profiles Polygenic Irritable Bowel Syndrome in Mestizo Individuals. Front. Cell. Infect. Microbiol. 10:72. doi: 10.3389/fcimb.2020.00072

INTRODUCTION

Irritable bowel syndrome (IBS) is a multifactorial disorder, caused by abnormalities within the gut-brain axis and resulting in autonomic hypersensitivity and gastrointestinal motility dysfunction (Distrutti et al., 2016). IBS is often accompanied by low-level inflammation, resulting from an increased interleukin (IL)18, IL1b, and myeloperoxidase effector production and activity (Aerssens et al., 2009; McKernan et al., 2011; Clarke et al., 2012). Although clinically divided into subtypes, evidence suggests that the genomic backgrounds that regulate neuro-immune traits and genomic differences among gut microbiota may underlie pathology and symptom display. Although gut microbiota varies throughout life, dysbiosis far exceeds the normal 10% variation range in IBS patients, with a decrease in butyrate producers, which favors the proliferation of Firmicutes and Proteobacteria phylum (Mayer et al., 2014). Currently, corticotropin-releasing factor (CRF), NOD-like receptor family pyrin domain containing 6 (NLRP6 or IDO1), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), Toll-like receptor 4 (TLR4), and cytochrome P450 1A (CYP1a) have been associated with low butyrate levels and colonic mucosal inflammation in humans and animal model of IBS, demonstrating the effects of genomic background on cross-signaling (Vujkovic-Cvijin et al., 2015; Wang et al., 2016; Layunta et al., 2018; Manzella et al., 2018; Martin-Gallausiaux et al., 2018; Zhao et al., 2018; Yu et al., 2019). Although hypothesis-free analyses, such as genome-wide association studies (GWAS) may be able to disentangle all possible interactions, a major drawback of IBS standardized trials is that most have been low-powered and primarily focused on participants with Caucasian ancestry (Gazouli et al., 2016). Previous reports have shown gene variants that are frequently associated with Amerindian inheritance, such as hepatocyte nuclear factor 4 (HNF4)-Diabetes Mellitus 2, and ulcerative colitis-associated, may widely diverge from HapMap (Wang et al., 2016; Granados-Silvestre et al., 2017). However, shotgun metagenome sequencing and analysis remains a fast and sensitive alternative for the exploration of gene network variants and microbiota phylogeny among a standardized cohort, providing the opportunity to explore both the microbial community and host interactions in IBS patients with particular genetic backgrounds (Sharpton, 2014).

The current research aimed to contribute to a deeper understanding of gene variations and single nucleotide polymorphisms (SNPs) in a Mestizo validation cohort of IBS patients and variations in the gut microbiota, at the genus taxonomic level, in close contact with the colonic mucosal lining.

METHODS

Study Population

Five IBS patients and 5 healthy controls (HCs) were recruited at the Hospital General de México Dr. Eduardo Liceaga, in Mexico City. This hospital is the largest general hospital of the Mexican public health system and serves patients that are referred from all over the country. Subjects of both sexes between 18 and 65 years old were included. All subjects completed the Rome III Adult Symptom Questionnaire. The IBS patients fulfilled the Rome III criteria and were further classified, according to their bowel habit subtypes, as IBS with diarrhea (IBS-D), IBS with constipation (IBS-C), mixed IBS (IBS-M), or unclassified IBS (IBS-U). HCs were recruited through invitations sent to the patients' relatives and advertisements. HCs did not meet the Rome III criteria for IBS or any other functional bowel disorder. Subjects who fulfilled the IBS criteria were also evaluated using the IBS severity scoring system (IBS-SSS) and were classified as mild (75 to <175), moderate (175–300), or severe (>300) (Francis et al., 1997; Almansa et al., 2011). The presence of organic gastrointestinal diseases, such as inflammatory bowel disease (IBD), celiac disease, and infectious gastroenteritis, or systemic diseases, such as diabetes, thyroid, autoimmune, or any coagulation disorders, was ruled out in all subjects. In addition, subjects were excluded if they received antibiotics or proton pump inhibitor treatment during the previous 3 months. All subjects underwent a complete colonoscopy, after bowel cleansing the evening before, using 4 sachets of polyethylene glycol/electrolytes dissolved in 4 liters of water (1 sachet/liter). Each sachet contained 105.0 g macrogol 3350, 1.43 g sodium bicarbonate, 2.80 g sodium chloride, and 0.37 g potassium chloride. Both the IBS patients and HCs reported to the Endoscopy Unit of the Hospital General de Mexico after an 8-h fasting period. After signing informed consent, they were given a colonoscopy under IV sedation. No patient had any colonoscopic abnormalities.

All subjects answered the Rome III Questionnaire and a Frequency of Food Intake Questionnaire, which was analyzed by Nutrients Analysis System (SNUT) software to determine the dietetic contents. Correlations between microbiota and dietary nutrients were conducted using Spearman's (Rho) and Pearson's (r) correlation coefficients, according to sample distribution. A $p \leq 0.05$ was considered to be significant.

This study was approved by the Ethics and Research commissions of the Faculty of Medicine at Universidad Nacional Autónoma de México (UNAM) (124/2014) and the respective commissions at the Hospital General de México (DI/15/UME/03/33).

Biopsy Sampling

Four biopsies were collected from the descending colon. The biopsies were placed into cryotubes, containing 500 µl RNAlater solution, and were transported at room temperature from the hospital to the Programa de Inmunologia Molecular Microbiana at the Faculty of Medicine at Universidad Nacional Autónoma de México (UNAM), where they were processed.

Genomic DNA Extraction and Sequencing

DNA (mean concentration 4.46 µg) was extracted from colon biopsies with the QIAamp® DNA mini kit (Qiagen, No.51306), following the manufacturer's instructions. The DNA quality was determined by agarose gel electrophoresis, and purity and quantity were determined by measuring 260/280 absorbance ratios (NanoDrop, ThermoFisher, USA).

Sequencing

DNA shotgun sequencing was performed on an Illumina HiSeq 4000 platform, at Macrogen Inc. (Seoul, Korea). Here, a TruSeq DNA PCR Free library (Illumina, USA) was constructed, and the sequencing was performed using a 2 × 100 bp paired-end format. Sequencing data were deposited at NCBI under the SRA accession number SUB6452398 and at BioProject ID PRJNA579180.

Sequence Processing

(1) *Sequence quality*. The sequences obtained from each sample (164,935,740–233,353,722 bp) were analyzed with FastQC software (v. 0.1.1.5), and low-quality sequences ($Q < 20$) were detected and removed. (2) *Merged reads*. The forward and reverse reads for each sample were merged, using PEAR software (v. 0.9.6), using the default settings, to increase the total length. (3) *Sequence alignment against the human genome*. All the samples were mapped with Bowtie software (v. 1.2.0), using the default settings, to align the reads against the human genome (Homo sapiens, GRCh38). On average, the number of non-aligned sequences returned 19,763,708 reads, whereas aligned sequences returned 193,086,669 reads, which were eliminated from the analysis.

Taxonomic Annotation

The sequences that were not aligned against the human genome were uploaded to the Metagenomics Rapid Annotation using Subsystems Technology server (MG-RAST), which compares the sequences against the non-redundant protein database M5nr (Keegan et al., 2016). We used the lowest common ancestor (LCA) method for taxonomic assignation, and mapped reads were considered based on the following parameters: an *e*-value 1e-5, minimum identity 60%, and 30-bp minimum alignment length. Metagenome annotations are available in MG-RAST, with the accession numbers 4683929.3, 4683933.3, 4683935.3, 4698475.3, 4700742.3, 4698476.3, 4702949.3, 4700744.3, and 4700745.3.

Data Management

The abundant MG-RAST profiles were transformed into relative frequencies for easier data handling. The relative abundances were plotted using phyloseqGraphTest and ggplot2 packages in R (v. 3.4.3). Finally, we conducted a statistical analysis of the relative frequencies of the gut microbiome genera relative to the identified SNPs, using the Mann-Whitney *U*-test in SPSS software (v. 12).

Genes and SNPs Associated With Gastrointestinal Disease

Variant annotation was performed using the Genome Analysis Toolkit (GATK), according to the Best Practices Pipeline published by the BROAD Institute (McKenna et al., 2010; van der Auwera et al., 2013). For data pre-processing, FASTQ files from our shotgun data were converted to BAM formats (not aligned) and aligned with the human genome (GRCh version 38 patch 13) using BWA (Li and Durbin, 2010). PCR duplicates were removed from reads files. Search variants (SNPs) were made with Haplotype Caller (van der Auwera et al., 2013). The SNPs identified in the samples were screened against known SNPs associated with intestinal bowel diseases (909 SNPs) and IBS (25 SNPs), based on the GWAS catalog, which was consulted in March 2019 and May 2019, respectively. Moreover, after reviewing the literature (RV), we identified 709 additional SNPs. In total, we identified 1,643 SNPs. Additionally, we conducted a search for genes previously associated with IBS and IBD, analyzing a total of 1,144 genes. Searches and annotations of variant information available in public databases were performed using Funcotator (Genome Annotation Toolkit v. 4.1.1.0) (Buniello et al., 2019). The IDs of SNPs were added using bcftools (v. 1.9) and dbSNP v. 1.1.1.1 (v. 151) (Sherry et al., 2001; Li, 2011; Kuhlwilm and Boeckx, 2019). We compared the presence of all identified SNPs between IBS patients and HCs, the SNPs that appeared differentially in at least three subjects between these groups were selected for further analysis. The **Figure 1** was created using R software V.3.6.0 with devtools package as well as ComplexHeatmap package (Gu et al., 2016), which allows the user to stack all levels of information onto one image. Tables with information about genes, SNPs, microbiome, genotypes, and disease were uploaded into the R environment and code was developed to arrange the information as seen on the **Figure 1**.

RESULTS AND DISCUSSION

Characteristics of the Study Subjects

The demographic and clinical characteristics of the population are shown in **Table 1**. IBS patients included 4 women and 1 man (mean age: 41 years), 3 who were classified as IBS-D, 1 as IBS-C, and 1 as IBS-M. HCs included 3 women and 2 men (mean age: 28 years).

Metagenomic Results

Among the taxonomic annotations of sequences not aligned with the human genome, sequences were classified as belonging to Archaea, Bacteria, Eukaryotes, and Viruses. A lower percentage of bacterial sequences was found in IBS patients (average 45.06%) compared with HCs (average 90.55%) (**Table S1**).

The most representative phyla were Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Verrucomicrobia, which were present in all metagenomes, with the exception of Fusobacteria, which was absent in one IBS patient (**Table 2**). Both Actinobacteria and Proteobacteria were found at higher frequencies in IBS patients compared with HCs. In contrast, the most representative phylum among HCs was Bacteroidetes. Significant increases in the Actinobacteria phyla ($p = 0.028$) and Proteobacteria ($p = 0.009$) and a significant decrease in Bacteroidetes ($p = 0.009$) was found in the IBS group compared with the HC group (**Table 2**). The genera found at higher relative frequencies in the metagenomes were *Bacteroides, Alteromonas, Neisseria, Streptococcus, Microbacterium*, and other previously described genera (**Figure 1A**). However, only *Bacteroides* was found in the metagenomes of all 10 subjects, with a higher relative frequency in HCs than in IBS patients. In the IBS group, significant increases in the relative frequencies of *Propionibacterium* ($p = 0.009$) and *Staphylococcus* ($p = 0.047$) and an increasing

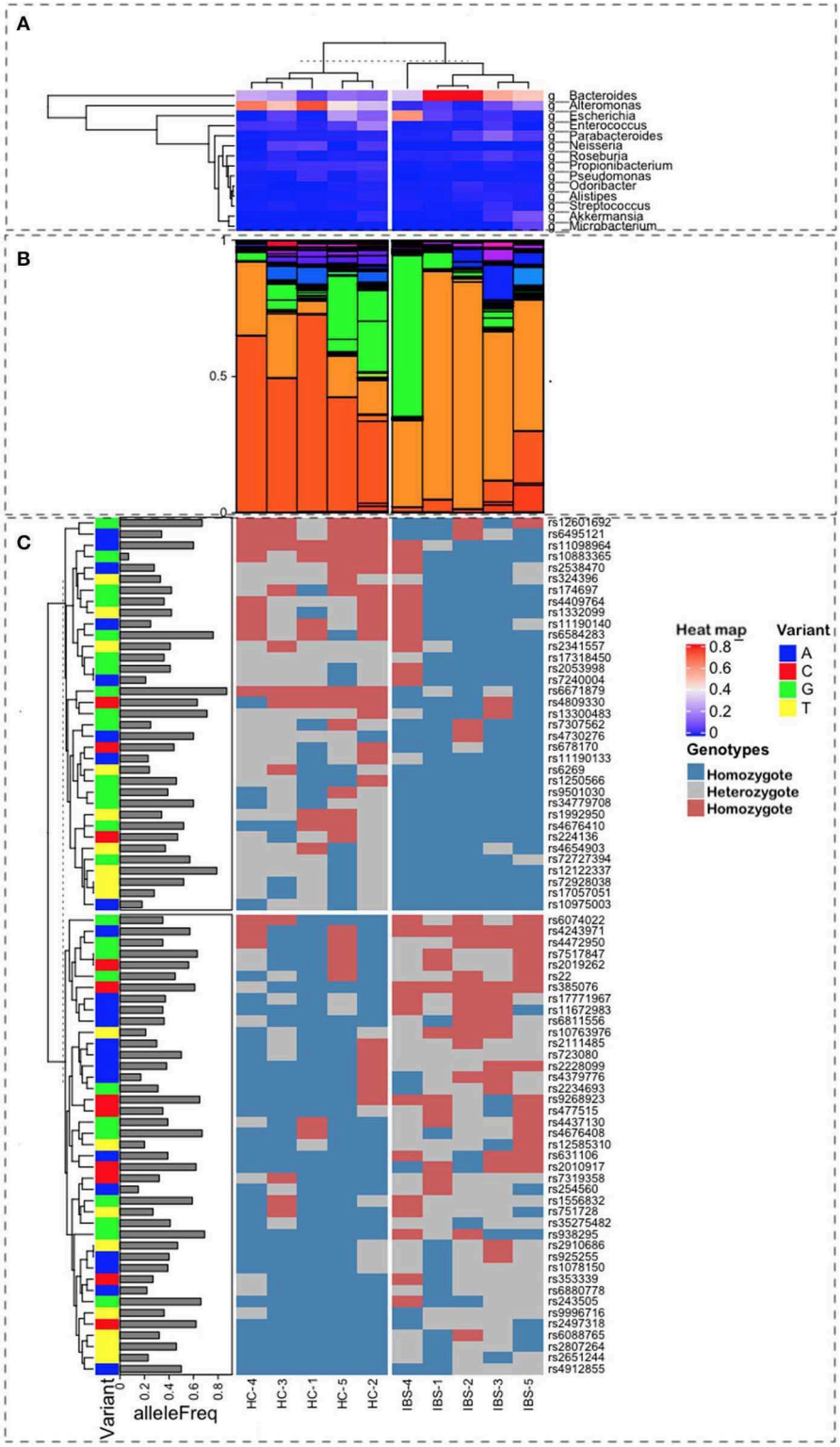

FIGURE 1 | Representation of the multiple sources of information used for this study. Ten samples were characterized, 5 as Healthy Control (HC), on the left-side plots, and 5 as Irritable Bowel Syndrome (IBS), on the right-side plots, with labels at the bottom of the figure. **(A)** Heatmap from a hierarchical clustering algorithm showing the level of presence for the top microbes at the genus level in both groups. Bacteroides are the most frequent in IBS, whereas the Alteromonas population is higher in HC samples **(B)**. Relative proportions of microbial taxa at the genus level show a clear difference in patterns between HC and IBS (light and dark orange). **(C)** Correlation with SNP variants associated with IBS and their allele frequencies in the Mexican population.

TABLE 1 | Demographic and clinical characteristics of the study population.

Sample	Sex	Age	Marital status	Educational level	BMI	Comorbidities	IBS subtype	Severity
IBS-1	W	41	Single	Middle school	Normal	None	IBS-C	Mild
IBS-2	W	60	Single	Technical	OW	None	IBS-D	Moderate
IBS-3	W	60	Widowed	Elementary	Normal	Hyperlipidemia	IBS-D	Moderate
IBS-4	W	37	Single	High school	Normal	None	IBS-D	Mild
IBS-5	M	24	Single	Higher degree	OW	None	IBS-M	Moderate
HC-1	M	24	Single	Higher degree	Normal	None	–	–
HC-2	W	33	Single	High school	Normal	None	–	–
HC-3	W	28	Single	Higher degree	Normal	None	–	–
HC-4	M	27	Single	Higher degree	OW	None	–	–
HC-5	W	44	Married	High school	OW	None	–	–

IBS, irritable bowel syndrome; C, constipation; D, diarrhea; M, mixed; HC, healthy controls; W, woman; M, man; OW, overweight.

TABLE 2 | Relative abundance per bacterial phylum in colonic biopsies.

Phylum	Relative abundance (%)										
	IBS					HC					
	1	2	3	4	5	1	2	3	4	5	*p-value*
Actinobacteria	9.0	2.9	2.9	1.8	4.6	1.6	1.5	0.8	0.47	0.49	0.028
Bacteroidetes	24.9	22.1	34.6	64.4	32.9	50.8	22.5	72.6	56.3	54.3	0.009
Firmicutes	23.4	27.5	42.3	28.2	44.3	35.4	25.3	23.0	38.3	42.1	0.917
Fusobacteria	0	20.4	0.16	0.31	0.10	0.13	0.70	0.01	0.04	0.09	0.347
Proteobacteria	42.4	26.6	19.8	5.18	17.4	11.9	22.9	3.33	4.40	0.18	0.009
Verrucomicrobia	0.18	0.27	0	0	0.41	0	0	0	0.39	1.21	0.914

IBS, irritable bowel syndrome; HC, healthy controls.

trend in the relative frequency of *Enterococcus* ($p = 0.076$) were observed compared with the HC group. In contrast, among HCs, *Dorea* ($p = 0.016$), *Faecalibacterium* ($p = 0.047$), and *Gemella* ($p = 0.041$) were significantly decreased compared with the IBS group (**Figure 1B**). We found no significant difference in Bifidobacterium between the IBS and HC groups. Interestingly, we found a higher frequency of the genus *Pseudomonas* among our IBS patients, which was determined by an increase only in the IBS-C and IBS-M patients, but not in the IBS-D patient.

The Polygenic Presence in IBS Patients, Based on SNPs

In animal models, the host genomic background has been demonstrated to be more important to the composition of gut microbiota than the microbiota found at points of contact with other microorganisms. In humans, GWAS analyses have identified relationships between host SNPs and gut microbiota. The relevant metabolic pathways include those associated with tryptophan metabolism, which are synthesized by microbiota components that regulate intestinal homeostasis. In our study, the combination of SNPs in the 5 IBS patients, based on allele frequency, was calculated for the Mexican Mestizo population. We selected 76 SNPs that showed differences (presence/absence) between IBS and HC groups. Of these, 40 SNPs were associated with the susceptibility for IBS. The hierarchical ordination showed two groups, consisting of high- and low-frequency SNPs among Mestizos (**Figure 1**, **Table S2**). Even those SNPs with the lowest frequency of 0.2 should be capable of having an effect. Although we only examined a subsample of SNPs, because these SNPs were shared among subjects, they demonstrate the different genomic backgrounds between HCs and IBS patients. The SNPs in IBS were associated, at the phylum level, with Firmicutes, Proteobacteria, and Actinobacteria, suggesting that the observed increase in Proteobacteria may be due to aerobic metabolic inflammation. Other studies, including ours, have identified SNPs in the tryptophan metabolic pathway that affect IgA levels, intestinal barrier formation, antimicrobial peptide production, and other immune activities due to dysbiosis resulting in decreased levels of aryl hydrocarbon receptor (*AHR*) ligands. In our IBS group, we identified the following SNPs: rs6495121, which is found in the *AHR*, which regulates toxicity via cytochrome *P450 1A1* (*CYP1A1*) and G-protein-coupled receptor 35 (*GPR35*); rs2228099, a candidate for type II diabetes and prediabetes intermediate traits (**Figure 1C** and **Table S1**). We found 7 SNPs, with frequency values of 0.2 alleles among the Mestizo population, including 4 that were associated with IBS risk factors, rs10763976, rs4379776, rs12585310, and rs254560; and 3 were associated with IBS protection, rs10883365, rs7240004, and rs10975003 (**Table 1**).

The SNPs Associated With the Central Nervous System in Irritable Bowel Syndrome (IBS)

The identified SNPs were integrated with what was already known about functional IBS diseases associated with the central nervous system (CNS) (**Table S1**). After the brain, the intestine is the second-most enervated organ in the body. The rs254560_AG SNP in the C5orf66 gene on chromosome 5 found in this study is similar to genes such as *CCND280*, *GLI381*, *or RB1CC182*, which have been described as regulatory CNS proteins that modify transcriptional activity and contribute to brain growth trajectory changes at different biological levels, ranging from cellular changes, brain organization differences and complex phenotypic traits (Kuhlwilm and Boeckx, 2019). The weights of common IBD risk alleles are significant determinants during chronic granulomatosis diseases (CGD) (Huang et al., 2016). Based on general linear models, gray matter (GM) alterations caused by SNPs and Early Adverse Life events (EALs) are predictive of IBS. These studies showed that catecholaminergic SNPs may interact with other genes and environmental factors, such as EALs, catechol-O-methyltransferase (*COMT*), and alpha-1D adrenergic receptors (*ADRA1D*) rs1556832 (Orand et al., 2015). In our study, we found that rs1556832 was heterozygous in IBS patients with mild and moderate symptoms. However, according to Orand et al. (2015), homozygous rs1556832 was associated with increases in the volume of somatosensory regions and the left precentral gyrus. In contrast, rs1556832 was significantly associated with GI symptom severity.

The GI tract contains approximately 95% of the total body serotonin content, which is also known as 5-hydroxytryptamine (5-HT) and is primarily synthesized by enteric endocrine cells (EECs). Enterochromaffin (EC) cells are the best-characterized subset of EECs and are the primary source of 5-HT in the gut (Kwon et al., 2019). EC cells are dispersed among epithelial cells in the mucosal layer of the GI tract and release 5-HT apically into the gut lumen and basolateral, in response to various mechanical and chemical stimuli. EC cells synthesize 5-HT from its precursor, L-tryptophan. Tryptophan hydroxylase (Tph) catalyzes the synthesis of 5-HT. Two isoforms of Tph enzymes regulate 5-HT synthesis, including Tph1, which is primarily found in EC cells, and dysregulated 5-HT signaling has been observed in patients with IBS. Because of the close proximity of gut microbiota and 5-HT producing EC cells in the gut mucosal layer, crosstalk between them is likely to play a critical role in the maintenance of intestinal homeostasis, and recently, gut bacteria have been shown to stimulate the release of 5-HT from EC cells. Two major bacterial phyla, Firmicutes and Bacteroidetes, and 5 minor bacterial phyla, Proteobacteria, Actinobacteria, Fusobacteria, Cyanobacteria, and Verrucomicrobia, comprise the gut microbiota in adult humans. According to *in vitro* studies, 5-HT affects the growth of *B. thetaiotaomicron* and *E. faecalis*, providing further support that 5-HT may be able to inhibit peroxisome proliferator-activated receptor (PPAR)-γ in a microbiota-dependent manner. Altogether, these findings suggest that 5-HT released from EC cells directly and indirectly (via modulation of b-defensin production) plays a crucial role in the regulation of the gut microbial composition.

Meanwhile, the *SLC2A9* gene was associated with an unclassified *Porphyromonadaceae* (for us only rs62295801) was identified, and these loci were associated with bacterial abundance. 5-HT strongly inhibits NOD2 expression, which may play a role in intestinal pathophysiology not only through its inherent innate immune role but also through interactions with other receptors, such as TLR2, and the modulation of the intestinal serotonergic system, by decreasing serotonin transporter (SERT) activity and expression (Layunta et al., 2018). Analysis of SERT activity showed NOD2 activation, suggesting that long-term NOD2 activation may decrease SERT expression via transcriptional and/or post-transcriptional mechanisms, offering an explanation for the observed reduction in 5-HT uptake (Layunta et al., 2018).

The Interaction Between the Immune Response and the Microbiome in IBS

The multidimensional relationship among key factors involved in vitamin D receptor (VDR) signaling (bile acids and ω6 fatty acids in particular) and the gut microbiota have been supported by genetic associations between functionally related loci, such as the HF4A rs9996716_AG SNP in the SHROOM3 gene on chromosome 4. The Proopiomelanocortin (POMC), shroom family member 3 (SHROOM3) *Marinilabiliaceae* family rs9996716_GA 5.58 × 10–9–0.690 has previously been demonstrated to increase the degradation of tryptophan along this immune responsive pathway during IBS and also found in our study (Wang et al., 2016). However, the relationship between TLR activation and kynurenine pathway activity in IBS remains unknown. A differential downstream profile of kynurenine production, subsequent to TLR activation, was found in IBS patients compared with healthy controls (Clarke et al., 2012). This profile included observed alterations in TLR1/2, TLR2, TLR3, TLR5, TLR7, and TLR8. An exaggerated response to TLR8 agonist by all cytokines investigated was observed in IBS patients. In addition, enhanced TLR2-induced tumor necrosis factor (TNF)- α release, TLR3-induced IL-8 release, TLR4-induced IL1b and TNF-α release, TLR5-induced IL1b and TNF-α release, and TLR7-induced IL-8 release were elevated in IBS-D patients (McKernan et al., 2011). The intestinal epithelial cells (IECs) play a central role in the host-microbiota dialogue by inducing the first microbial-derived immune signals.

Among Firmicutes, the most active genera for indoleamine 2, 3-dioxygenase (IDO)-1 expression were *Clostridium, Lachnoclostridium, Ruminoclostridium,* and *Roseburia*, according to principal component analysis (PCA) and correlation analyses on short-chain fatty acid (SCFA) concentrations. *Clostridium butyricum* regulates the visceral hypersensitivity of IBS (Shon et al., 2015; Vujkovic-Cvijin et al., 2015; Martin-Gallausiaux et al., 2018). Approximately 40% of the colorectal biopsy specimens from IBS patients showed non-specific inflammatory manifestations, such as neutrophils, mast cells (MCs), and T cell infiltration, which is often referred to as low inflammation. Caspase-1, ASC, and CARD8 form NLRP, NF-κB, IL-1β, IL-18.

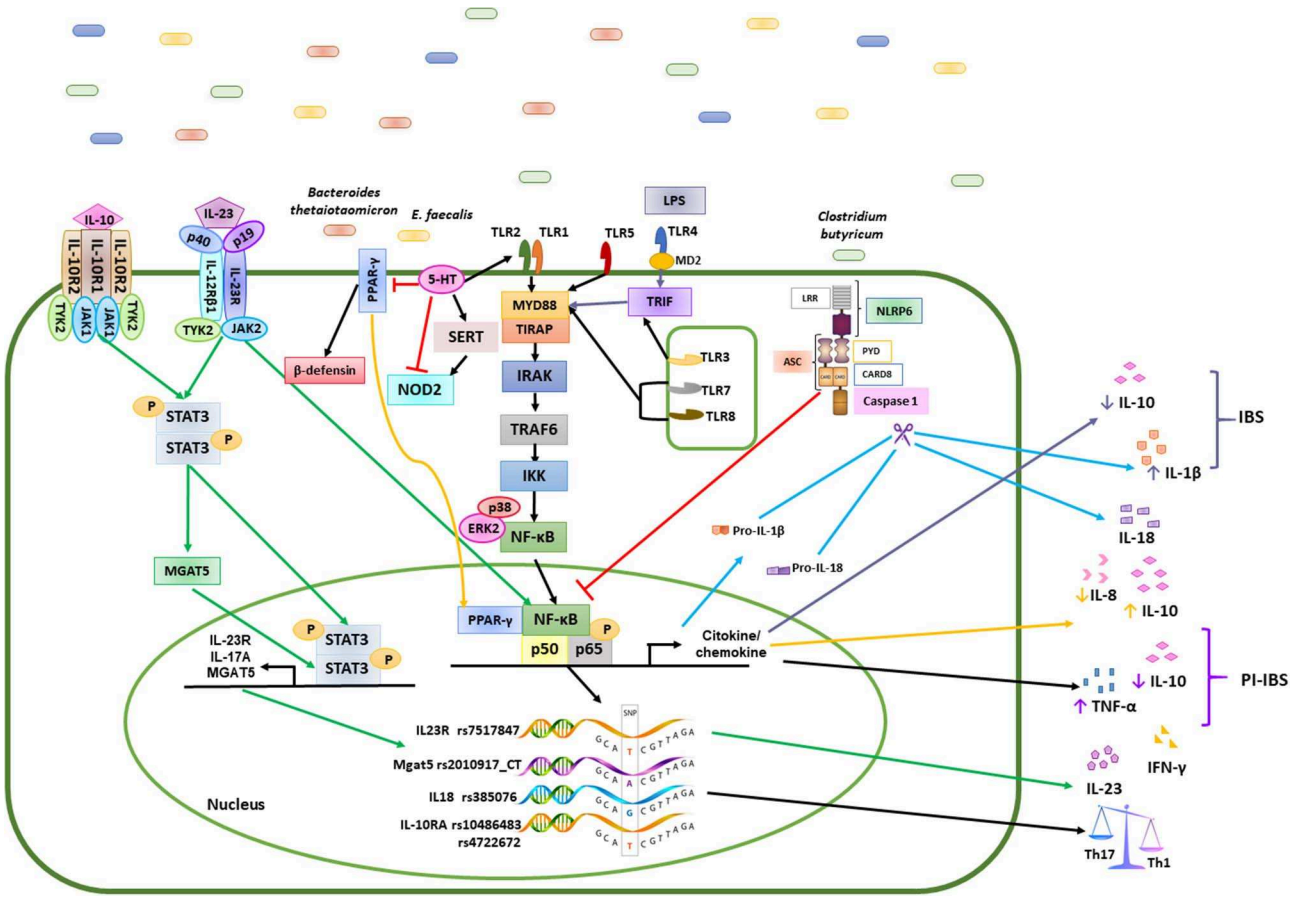

FIGURE 2 | Mechanisms of the immune response induced by polygenic diseases related to the composition of the intestinal microbiota in patients with IBS and healthy subjects. Bacteria such as Bacteroidetes, Clostridium, *E. faecalis*, and Firmicutes, promote the activation of different signaling pathways in the epithelial cells of the intestinal mucosa through various mechanisms, while commensal bacteria stimulate the induction of a Th1/Th2 response by the production of cytokines such as IL-1β, IL-6, IL-10, IL-12, and TNF-α in healthy controls (HC). Differentiation of antigen-specific Th17 cells can be promoted by the increase in cytokine levels such as IL-1β, IL-8, IL-10, IL-17, IL-18, IL-23, TNF-α and INF-γ in patients with IBS. the green arrows represents the signaling pathway that is activated for the production of IL-23; the yellow arrows are the signaling pathway for the production of IL-8 and IL-10 in patients with IBS; the black arrows represents the path of activation of NFκB in healthy subjects; the blue arrows represent the caspase activation pathway and the production of IL1β and IL18; The truncated red line represents the inhibition of transcription factors or other molecules.

Clostridium butyricum might play a beneficial role in visceral hypersensitivity of IBS by inhibiting low-grade inflammation of colonic mucous through its action on NLRP6 (Zhao et al., 2018). Polymorphisms in cytokine-encoding genes and alterations in allele and genotype frequencies have been reported in IBS patients and may result in changes in the production of some cytokines. SNPs in the IL-6 region have been associated with an increased risk of developing PI-IBS. These SNPs were identified as independent risk factors for developing PI-IBS (Lazaridis, 2018).

CXCL14 effectively promotes chemotaxis of immature, but not mature, DCs, both *in vivo* and *in vitro* (Tanegashima et al., 2010; Lu et al., 2016). Interestingly, CXCL14 shares striking common structural characteristics with them, including large, positively charged patches on its molecular surface, three anti-parallel β-strands, similar to those found in β-defensin, and a C-terminal α-helix that is typical for cathelicidin LL-37. *Enterococcus, Rosseburia, Propionibacterium, Streptococcus,* and *Microbacterium*, which were found in this study suggested that CXCL14 and human β-defensin described to have activity against Gram positive coccoid suggest an important question to be solved (**Figure 2**). Specifically, CXCL14 exhibits antimicrobial activity against Gram-negative *E. coli*, Gram-positive *Staphylococci* species, although a *Propionibacterium*, *Pseudomonas aeruginosa*, and *Streptococcus* species, which were found in this study needs to be described (Lu et al., 2016). The expression of *Cxcl14* in the CNS and the changes in neuropeptides, in addition to the metabolic changes described previously, suggested an important role for *Cxcl14* in the coordination of feeding behavior (Hernández-Ruiz and Zlotnik, 2017).

Because two SNPs (rs9268923_CT identified in this study, on chromosome 6 but not rs117782746) overlapped between CD and IBD although, the total number of unique identified SNPs was 64. Likewise, Nod2$^{-/-}$ mice displayed higher expression levels of IFN-γ in their ileum but lacked signs of colitis and prolapse development. The scope and nature of gene-smoking

interactions in IBD may be exemplary for other diseases. A focused analysis of the human leukocyte antigen (HLA) region revealed that only a subset of 16 IBD-predisposing alleles was found to interact with smoking. Several SNPs in the HLA region were also found to interact with smoking in relation to either CD, UC, or IBD (Yadav et al., 2017).

Binding to IL-23 receptor (IL-23R) complex encoded by the rs7517847 SNP gene on chromosome 1, was found in this study significantly increased the Janus kinase (JAK)-STAT and NF-κ B signaling pathways. IL-23/IL23R signaling plays critical roles in innate and adaptive inflammatory responses in the intestinal mucosa. As described previously, evidence indicated an association near the Smad7 gene, which encodes a protein that is overexpressed in IBD patients (Damas et al., 2017). This locus is relevant to IBS risk in the Hispanic population and may lead to a better understanding of the genetic architecture across this diverse population.

Correlation Between Nutrients and Colonic Microbiome

Recently, we analyzed the correlation between nutrients and the microbiome and compared the results between IBS patients and HCs (Cano-Manrique et al., 2018). The results showed a higher intake of Fe (0.96 ± 0.1 vs. 0.61 ± 0.3 mg, $p = 0.05$) and vitamin B12 (5 ± 2.1 vs. 2.7 ± 0.7 mg, $p = 0.04$), suggesting a more animal-based diet in IBS patients. Significant correlations between CM and nutrients varied between the IBS and HC groups. In IBS, the phylum Proteobacteria correlated with Mn ($p = 0.023$) and monounsaturated fatty acids (MUFAs) ($p = 0.03$), possibly as a result of an animal-based diet, whereas in HCs, Proteobacteria correlated with lactose ($p < 0.001$). The genus *Ruminococcus*, a plant polysaccharide-utilizing bacterium, negatively correlated with cholesterol ($p = 0.01$) in IBS patients. *Faecalibacterium* correlated positively with cholesterol ($p = 0.03$) in IBS patients and negatively correlated with MUFAs in HCs ($p = 0.03$). *F. prausnitzii*, which was significantly decreased in IBS patients, was not correlated with any nutrients in IBS patients, whereas in HCs it was negatively correlated with CHO, glucose, and starch, possibly due to the fermentation of the starch during butyrate production.

CONCLUSION

This study provides new evidence that 76 SNPs with differing penetrance might be involve in the susceptibility of IBS following a trend previously described for IBD. Since high frequency are often downgraded, the lowest 7 SNPs with allelic frequency among the Mestizo population could drive to dysbiosis. Of these, 2 SNPs were associated with IBD, 2 SNPs were associated with ulcerative colitis, 2 SNPs are intronic in the PARD 3 gene, associated with the regulation of serotonin, and the last SNP has previously been associated with IBS. We also found an enrichment of the Firmicutes, Proteobacteria, and Actinobacteria phyla, where enterobacteria, enterococcus, Akkermancia, Alteromonas, and others play important roles in the pathophysiology of polygenic IBS patients, associated with SNPs in TLRs, IL18, IL23, and IL17, which can acts as a pro or anti-inflammatory effect depending on environment. This enrichment may be associated with inflammatory regulatory activities, including reduced IL-10 levels and the inhibition of b-defensin production from colonic epithelial cells, which altogether results in the perpetuation of gut inflammation. In IBS, compared with controls, different correlations exist between SNPs, the colonic microbiome, and nutrients (**Figure 2**).

DATA AVAILABILITY STATEMENT

Sequencing data were deposited at NCBI under the SRA accession number SUB6452398 and at BioProject ID PRJNA579180.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comisión de Investigación-ética, Facultad de Medicina, UNAM 125/2014. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RA-H contributed to SNPs analysis and reviewed the manuscript. MS contributed to study concept, patient recruitment, grant support, and manuscript. PO performed pipeline for DNA extraction, contributed to the metagenomic analysis, and reviewed the manuscript. GL-L performed initial analyses and structured the analysis pipeline for metagenomes. RS-C performed DNA extraction and metagenomic analyses. A-MZ recruited patients and conducted colonoscopies and biopsies. LA contributed to analysis of pipelines for the metagenomes. GA-F contributed to the analysis and design of figures and reviewed the manuscript. AV performed statistical analyses. SP contributed to study concept, coordination, supervision, and reviewed the manuscript. YL-V contributed to study concept, coordination, supervision, analyses, grant support, and wrote and reviewed the manuscript. MC contributed to structure the analysis pipeline for the metagenomes.

FUNDING

This study was financed by grant PAPIIT IV200315 from PAPIIT-UNAM and Fundación Gonzalo Río Arronte I.A.P (No.Reg. S.590), research support from the Research Division of the Medicine Faculty at UNAM, and Fundación Miguel Aleman. We thank ASOFARMA México for providing polyethyleneglycol/electrolytes for the subjects' bowel cleansing.

ACKNOWLEDGMENTS

The Consorcio Mexicano para el Estudio del Microbioma Humano includes MC, RA-H, GL-L, YL-V, PO, SP, RS-C, E. Martínez-Romero (Centro de Ciencias Genómicas, UNAM), A. I. Castillo-Rodal, G. Castillo-Rojas, A. R. Villa-Romero (Facultad de Medicina, UNAM), L. D. Alcaraz-Peraza, A. C.

Becerra-Bracho, J. A. Campillo-Balderas, A. E. Lazcano-Araujo (Facultad de Ciencias, UNAM), A. Frank-Hoeflich (Centro Ciencias de la Complejidad, UNAM), D. I. Piñero-Dalmau (Instituto de Ecología, UNAM), F. Bernal-Sahagún, Vanessa Fuchs-Tarlovsky, Nutricionist, J. C. López-Alvarenga, MS, A-MZ (Hospital General de México), J. J. Calva-Mercado (INCMNSZ), V. M. Bautista de Lucio, E. Graue-Hernandez, J. L. Pedro-Aguilar (Instituto de oftalmología Conde de Valenciana) and Fernando Bernal Sahagún. We are grateful to Tania Hernández Montiel, Cesar David Mejía Rueda, Juan Manuel Pérez Viquez, and Ricardo Rojas Rubin for their support in patient enrollment for the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00072/full#supplementary-material

Figure S1 | Relative frequency of the genera in the analyzed metagenomes. Taxonomic differences at genus level in IBS and HC metagenomes. The most frequent genera in HC were *Bacteroides, Faecalibacterium, Ruminococcus*, and *Prevotella* to a lesser degree. In IBS patients, there is an increase in *Enterococcus, Bifidobacterium,* and *Acinetobacter* in sample IBS-1C.

Table S1 | List of SNPs with differences between IBS and HC groups.

Table S2 | Genes associated to SNPs of **Table S1**.

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Conflict of Interest: SP is an Advisor for Becton-Dickinson and A-MZ is an advisor for Olympus. MS received grant support from Alfa-Sigma Mexico and Genova Diagnostics; advisory Board from Alfa-Sigma Mexico and Gemelli Biotech; consultant of Commonwealth Diagnostics International Inc.; speaker for Alfa-Sigma Mexico, Commonwealth Diagnostics International Inc., Mayoly-Spindler Mexico, and Takeda Mexico.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

BRIEF RESEARCH REPORT
published: 29 October 2020
doi: 10.3389/fcell.2020.582320

Microbiome Profiling Reveals Gut Dysbiosis in the Metabotropic Glutamate Receptor 5 Knockout Mouse Model of Schizophrenia

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Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: 11 July 2020
Accepted: 08 October 2020
Published: 29 October 2020

Citation:
Gubert C, Kong G, Uzungil V, Zeleznikow-Johnston AM, Burrows EL, Renoir T and Hannan AJ (2020) Microbiome Profiling Reveals Gut Dysbiosis in the Metabotropic Glutamate Receptor 5 Knockout Mouse Model of Schizophrenia. Front. Cell Dev. Biol. 8:582320. doi: 10.3389/fcell.2020.582320

Schizophrenia (SZ) is a psychiatric disorder that constitutes one of the top 10 global causes of disability. More recently, a potential pathogenic role for the gut microbial community (microbiota) has been highlighted, with numerous studies describing dysregulated microbial profiles in SZ patients when compared to healthy controls. However, no animal model of SZ has previously recapitulated the gut dysbiosis observed clinically. Since the metabotropic glutamate receptor 5 (mGlu5) knockout mice provide a preclinical model of SZ with strong face and predictive validity, in the present study we performed gut microbiome profiling of mGlu5 knockout (KO) and wild-type (WT) mice by 16S rRNA sequencing of bacterial genomic DNA from fecal samples, analyzing bacterial diversity and taxonomic composition, as well as gastrointestinal parameters as indicators of gut function. We found a significant genotype difference in microbial beta diversity. Analysis of composition of microbiomes (ANCOM) models were performed to evaluate microbiota compositions, which identified a decreased relative abundance of the *Erysipelotrichaceae* family and *Allobaculum* genus in this mouse model of SZ. We also identified a signature of bacteria discriminating between the genotypes (KO and WT), consisting of the Erysipelotrichales, Bacteroidales, and Clostridiales orders and macroscopic gut differences. We thus uncovered global differential community composition in the gut microbiota profile between mGlu5 KO and WT mice, outlining the first evidence for gut dysbiosis in a genetic animal model of SZ. Our findings suggest that this widely used preclinical model of SZ also has substantial utility for investigations of gut dysbiosis and associated signaling via the microbiota–gut–brain axis, as potential modulators of SZ pathogenesis. Our discovery opens up new avenues to explore gut dysbiosis and its proposed links to brain dysfunction in SZ, as well as novel therapeutic approaches to this devastating disorder.

Keywords: gut dysbiosis, microbiota, microbiome, psychiatric disorders, schizophrenia, mGlu5 knockout mice

INTRODUCTION

Schizophrenia (SZ) is a devastating psychiatric disorder characterized by positive (e.g., hallucinations) and negative (e.g., reduced motivation) symptoms, and cognitive deficits, which existing pharmacological treatments generally fail to comprehensively treat (Leung et al., 2019). While it affects both men and women, there are sex differences in SZ, with men showing an earlier age at onset together with higher propensity to negative symptoms, co-morbid substance abuse and lower social functioning (reviewed in Li et al., 2016). Importantly, chronic gastrointestinal (GI) tract issues exist as comorbid symptoms of SZ, including gut inflammation (Severance et al., 2015; McCutcheon et al., 2020).

More recently a potential role for the microbial community that resides in the gut (called gut microbiota) in SZ pathogenesis has been highlighted, with multiple groups describing dysregulated microbial profiles in SZ patients when compared to healthy controls (Castro-Nallar et al., 2015; Schwarz et al., 2018; Shen et al., 2018; Xu et al., 2019; Szeligowski et al., 2020). Disruption of microbiota can lead to gut microbial imbalance, called gut dysbiosis, and this condition has been shown to occur in various brain disorders, including SZ (Cryan and Dinan, 2012). Gut dysbiosis not only means a differential gut microbial population constituting a pathogenic profile but is also associated with impairment in gut integrity, functionality, intestinal permeability, as well as gut inflammation, and thus an aberrant gut environment. This profile generates a milieu of signaling molecules that ultimately can communicate with the brain through neural communication (via the vagus nerve), endocrine signaling, the immune system and microbial metabolites, thus modulating brain function, and, most remarkably, cognition (reviewed in Gubert et al. (2020)).

Due to discrepancies between clinical studies regarding specific taxa abundance and enrichment, a unique gut microbiome profile for SZ patients is still not determined (Macedo e Cordeiro et al., 2020). However, several clinical studies using different approaches in order to determine the SZ microbiota profiling or signature by determining the population diversity and taxonomic composition or bacterial abundance have been performed, revealing a potential new etiological aspect of SZ associated with an overall gut dysbiosis (reviewed by Rodrigues-Amorim et al., 2018). In fact, recent studies suggest that the disruption of the microbiota–gut–brain axis may promote the development of SZ and additionally, a few bacterial taxa including *Veillonellaceae* and *Lachnospiraceae* may associate with SZ severity (Zheng et al., 2019). Further evidence that SZ microbiota may be disease-causing is provided by a metagenome-wide association study showing differences in short-chain fatty acids (SCFAs), and neurotransmitter metabolism, synthesis and degradation in patient microbiota (Zhu et al., 2020). Given the emerging evidence supporting a role for gut dysbiosis in SZ pathogenesis, there is great potential to exploit this as an intervention target, as microbiota can be modulated via a range of therapeutic approaches.

Mice are frequently used to probe mechanistic questions relating to complex brain disorders, including in gut microbiota research (Nguyen et al., 2015). In several studies, fecal matter transplant of gut microbiota from SZ patients has been reported to result in behavioral changes in mice reminiscent of SZ symptoms (Zheng et al., 2019; Zhu et al., 2019, 2020). Interestingly, germ-free mice receiving SZ microbiome fecal transplants demonstrated lower glutamate and higher glutamine and GABA while SZ-relevant behavior in mice (Zheng et al., 2019). However, despite clinical evidence, no animal model of SZ has previously modeled gastrointestinal dysfunction or dysbiosis, although gut dysbiosis has been characterized in animal models of other psychiatric disorders, such as depression (Cheung et al., 2019; Sun et al., 2019).

In the present study, we aimed to profile the gut microbiome of a well characterized mouse model of relevance to SZ, metabotropic glutamate receptor 5 (mGlu5) knockout mice and their wild-type (WT) controls (Gray et al., 2009; Burrows et al., 2015; Zeleznikow-Johnston et al., 2018). Mouse models of schizophrenia are scrutinized against construct, face and predictive validity. While construct validity, or how well an animal model recapitulates the etiology, is difficult to achieve given the complex genetic and environmental drivers of SZ, mGlu5 KO mice model glutamatergic disruption hypothesized to contribute to the disorder (Devon et al., 2001; Gupta et al., 2005) and modulators of the receptor have been of interest in the context of treatment (Balu et al., 2016; Maksymetz et al., 2017; Stansley and Conn, 2018; Uno and Coyle, 2019). The mGlu5 KO mouse model exhibits abnormal brain maturation (Hannan et al., 2001) and a behavioral phenotype that mimics the positive and cognitive symptoms of SZ. Specifically this includes impairments in pre-pulse inhibition (a translational measure of sensorimotor gating in SZ patients and mice), hyperlocomotion (proxy for psychomotor agitation in psychosis) and short-term and long-term spatial memory deficits (Brody and Geyer, 2004; Brody et al., 2004; Gray et al., 2009; Xu et al., 2009; Bird et al., 2014; Burrows et al., 2015; Zeleznikow-Johnston et al., 2018; Lim et al., 2019; Aguilar et al., 2020). Predictive validity refers to response to a clinically effective treatment and beneficial effects on both positive and cognitive symptoms have been shown in mGlu5 mice following treatment with the antipsychotic drug clozapine (Gray et al., 2009).

To date, no study has scrutinized gut health in mGlu5 KO mice or any other mouse model of relevance to SZ, and we hypothesized that differences in the gut microbiome would exist between the two genotypes. To characterize the general gut health, we have performed microbiome profiling using 16S rRNA sequencing, analyzing bacterial diversity and taxonomic composition, as well as gastrointestinal parameters as indicators of gut function. The overall goal of this study was to establish whether this SZ mouse model could be utilized as an appropriate tool for preclinical study of gut dysbiosis.

MATERIALS AND METHODS

Animal Husbandry

Wild-type (WT) and mGlu5 KO male mice (Grm5tm1Rod; Lu et al., 1997) were generated from heterozygous breeding

pairs that had been maintained past generation F10 on a C57Bl/6 background. Genotypes were determined by PCR, from a tail biopsy. The mice were co-housed according to genotype after weaning at 4 weeks of age, in order to avoid potential sharing of microbiota due to the coprophagic nature of mice, unless indicated. The housing condition consisted of open-top standard mouse cages (34 × 16 × 16 cm^3; 2–4 mice/box) with basic bedding and nesting materials. Cage changes were performed weekly.

For 16S rRNA sequencing and body weight assessment the same cohort of mice was used, with a total of 6 mGlu5 KO and 6 WT mice, both genotypes split between two cages. All mice had *ad libitum* access to food and water and were housed in a controlled room of 22°C of temperature and 45% of humidity on a 12:12 h light/dark cycle. All procedures were approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics Committee and were performed in accordance with the relevant guidelines and regulations of the National Health and Medical Research Council Code of Practice for the Use of Animals for Scientific Purposes.

Food and Water Intake

Two extra cohorts were generated and used to assess food and water intake. While for the water intake measures, those mice were still separated by genotype, for food intake measures the respective mice were housed in mixed genotype groups (2–4 mice per box). Cumulative food and water intake were assessed, both over 4 weeks in grouped-housed mice, with the intake normalized to body weight to account for variability. These results are represented as mg of food per g of body weight and as g of water per g of body weight, respectively, to cumulative food and water intake.

Gastrointestinal Measurements

A separate cohort of 7 WT and 6 mGlu5 KO mice at 26 weeks of age was used in order to determine gastrointestinal parameters.

Fecal Output and Fecal Water Content

Fecal output and fecal water content were assessed by single caging the animals for 1 h and counting the number of fecal pellets expelled during this period. All the fecal pellets were collected, and the total weight was measured before being dried at 95 °C for > 3 h. The percentage of difference between the initial total feces weight and the dry weight is taken as fecal water content.

Gastrointestinal Transit Time

Gastrointestinal transit time was determined using the non-absorbable red dye Carmine red (Sigma-Aldrich), prepared as a 6% (w/v) solution in 0.5% methylcellulose (Sigma-Aldrich) filtered and autoclaved prior to use. Non-fasted mice were gavaged with 150 μL of the carmine solution and housed individually. The time taken from gavage to the first appearance of carmine in the feces was recorded as the total transit time.

FITC-Dextran Intestinal Permeability

Intestinal epithelial barrier permeability was assessed by 4 kDa fluorescein isothiocyanate (FITC)−dextran (Sigma-Aldrich). Mice were fasted for 4 h prior to oral gavage with 150 μL FITC-dextran (dissolved in PBS to a concentration of 100 mg/mL). Blood was collected 4 h post administration, and immediately transferred to an EDTA collection tube and further centrifugated at 1,000 × g for 10 min. Plasma was collected and fluorescence was quantified at an excitation wavelength of 485 nm and 528 nm emission wavelength (PHERAstar *FSX*, Millipore). Serial dilutions of FITC-dextran in PBS were used to calculate a standard curve.

Macroscopic Assessment

Mice were euthanized by cervical dislocation, and their intestines were excised. The intestine was placed in a non-absorbent surface and the length of cecum and colon was measured using a ruler. The weight of cecum with their contents was also evaluated. All of these parameters were normalized to the body weight (g) of the animals, unless indicated.

Fecal Sample Collection and DNA Extraction for 16S rRNA Sequencing

At 12 weeks of age, fecal samples were collected. Mice were individually placed in clean cages for up to 10 min. Fresh pellets were collected and immediately frozen in dry ice and stored at −80°C until further processing.

The fecal genomic DNA was extracted using the PowerSoil HTP kit (Qiagen). The number of sequences per sample was determined as the standard quality control metric (**Supplementary Table 1**), which showed a mean of approximately 75,500 sequences across all the samples. The extracted genomic DNA and amplified using universal prokaryotic 515F and 806R primers targeting the V4 hypervariable region of the 16S small subunit rRNA gene (Caporaso et al., 2011). Amplicon 16S rRNA gene sequences were generated using paired-end 150 bp sequencing on the Illumina MiSeq platform.

16S rRNA Sequencing, Bioinformatics, and Statistical Analysis

Illumina MiSeq raw FASTQ data were processed using Qiita for quality control, demultiplexing sequences, trimming, and resolving exact sequence variants (ESVs) or Amplicon Sequence Variants (ASVs) with Deblur (Amir et al., 2017). The representative sequences were mapped onto Silva_132 99% to obtain the taxonomic identity of ASVs (Quast et al., 2012). Downstream analyses were computed using R software version 3.5.2.

We first measured the alpha-diversity, which summarizes the diversity of microbial structure within a sample. Reads were rarefied to 1100 reads (**Supplementary Figure 1**), which is the global minimum of the number of reads in the sequencing data, to compute several alpha-diversity metrics, including species richness (Observed), Shannon, Inverse Simpson and Fisher metrics, using the "Phyloseq" R package (McMurdie and Holmes, 2013). Species richness (Observed) is the number of operational taxonomic units (OTU) observed in a given sample. Shannon, Inverse Simpson and Fisher diversity metrics are a composite

measure of richness (number of OTUs present) and evenness (relative abundance of OTUs). Kruskal–Wallis test was used to compare the species richness and alpha-diversity measurements between the genotypes.

To estimate beta-diversity, which summarizes the diversity between samples, the counts were normalized to their relative abundance by dividing raw counts from a particular sample by the total number of reads in each sample. We applied a relative abundance cut-off of 0.01% on the data (including only the relative abundance $>$ 0.01% of the data detected), resulting in a total of 202 ASVs for the subsequent analysis. Two measures of beta-diversity, Bray-Curtis and unweighted UniFrac distances (Lozupone and Knight, 2005), were calculated and used in principal coordinates analysis (PCoA). The unweighted UniFrac distance accounts for the phylogenetic relationship between the OTUs, whereas the Bray-Curtis distance accounts for the abundance of the OTUs. To determine whether the visually observed differences were statistically significant, Adonis (Permutation multivariate ANOVA—PERMANOVA) from the "vegan" R package was performed with 999 permutations (Anderson, 2001; Dixon, 2003). The R^2 value reported by Adonis PERMANOVA indicates the amount of variance, on a scale of 0 to 1, in the data which can be explained by the factors tested.

Before testing for differential abundance in the various taxonomic levels, centered log-ratio (CLR) transformation was applied to account for compositionality in microbiome data. Analysis of composition of microbiomes (ANCOM) was used to identify differential relative abundance of each bacterial family and genus between genotypes (Mandal et al., 2015). ANCOM accounts for the compositional nature of the taxa relative abundances and reside on the analysis of difference in pairwise log-ratio while controlling for false discoveries. We applied ANCOM with FDR correction of 0.05. A high "w score" generated by this test indicates the greater likelihood that the null hypothesis can be rejected, indicating the number of times a parameter is significantly different between groups.

Moreover, sparse PLS discriminant analysis (sPLS-DA) from the "mixOmics" package in R was used to identify a signature of discriminative ASVs associated with genotype (Lê Cao et al., 2016; Rohart et al., 2017). The performance of the model is evaluated using the leave-one-out cross validation method.

Co-occurrence Network Analysis

Given that microbiome data is compositional and transformation from absolute to relative abundances introduces spurious correlations, Sparse Correlations for Compositional data (SparCC) was employed to calculate correlations between ASVs to build the co-occurrence network (Friedman and Alm, 2012). SparCC and calculation of two-sided pseudo p values were performed based on bootstrapping of 100 repetitions. Correlations with p-values of < 0.05 and magnitude of = 0.6 (indicating strong co-abundance relationships) or =−0.6 (indicating strong exclusion relationships) were used to construct a network using qgraph (Epskamp et al., 2012). Clusters within the network were determined using the walktrap algorithm with 4 steps implemented in igraph (Csardi, 2006). The network was then visualized and plotted in Cytoscape 3.8 using a customized layout (Shannon, 2003).

General Statistical Analysis

In addition to the gut microbiome analyses described above, to verify the effects of genotype on the body weight, food intake, and water intake, unpaired *t*-tests were performed. These analyses and respective graphs were constructed using Graph® Pad Prism 8 software. In all cases, significance level was set to $p < 0.05$.

RESULTS

Gut Dysbiosis in mGlu5 KO Mice

To characterize the gut microbiome, the genomic DNA from feces collected at 12 weeks of age was extracted for 16S amplicon sequencing. Estimates of alpha and beta diversity, both indicative of differences in microbial communities, were obtained using a bioinformatic approach to cluster genetic data from mGlu5 KO and WT mice samples.

The alpha diversity indices (Observed, Shannon, Inverse Simpson, and Fisher) were calculated and plotted to visualize the difference between the two genotypes. Further statistical testing using Kruskal–Wallis method revealed no significance in any of the alpha diversity indices measured [**Figure 1A**, Kruskal Wallis test, Observed (H = 2.08, df = 1, p = 0.1488); Shannon (H = 0.1, df = 1, p = 0.7488); Inverse Simpson (H = 0.1, df = 1, p = 0.7488); Fisher (H = 2.08, df = 1, p = 0.1488)].

For beta diversity, both Bray-Curtis and unweighted UniFrac distance were calculated and the samples were ordinated based on those distances. When the entire population was examined together on a PCoA plot, using both the Bray-Curtis and unweighted UniFrac distance, samples tended to cluster according to the genotype. For Bray-Curtis PCoA, the stratification of samples according to groups was mostly on the second component (Axis 2), accounting for approximately 17% of variation in the data (**Figure 1B**; Bray Curtis dissimilarity distance ${}_{Permanova}R^2$ = 0.19, p = 0.021). Whereas for unweighted UniFrac PCoA, the stratification of samples according to groups was mostly on the first component (Axis 1), accounting for approximately 32% of variation in the data (**Figure 1C**; unweighted UniFrac distance ${}_{Permanova}R^2$ = 0.23, p = 0.003).

Across all mice, the most abundant Phyla was Bacteroidetes (74.9%), followed by Verrucomicrobia (14.6%), and then the Firmicutes (9.0%), which together made up approximately 98.5% of total abundance (**Figure 2A**). At Class level, the most abundant were Bacteroidia (74.8%), Verrucomicrobiae (14.5%), Clostridia (5.5%) and Erysipelotrichi (3.5%), which together are 98.3% of total abundance (**Figure 2B**). In addition, the most abundant Order were Bacteriodales (74.8%), Verrucomicrobiales (14.5%), Clostridiales (5.5%) followed by Erysipelotrichales (2.6%) (**Figure 2C**). A total of 27 bacterial families were detected in genomic data, and 23 remained after passing through a relative abundance cut-off of 0.01% (**Figure 2D**). The most abundant bacterial Family in its turn was *S24.7* (from Bacteriodales order), comprising 51.5% of the bacterial population, followed by *Verrucomicrobiaceae*

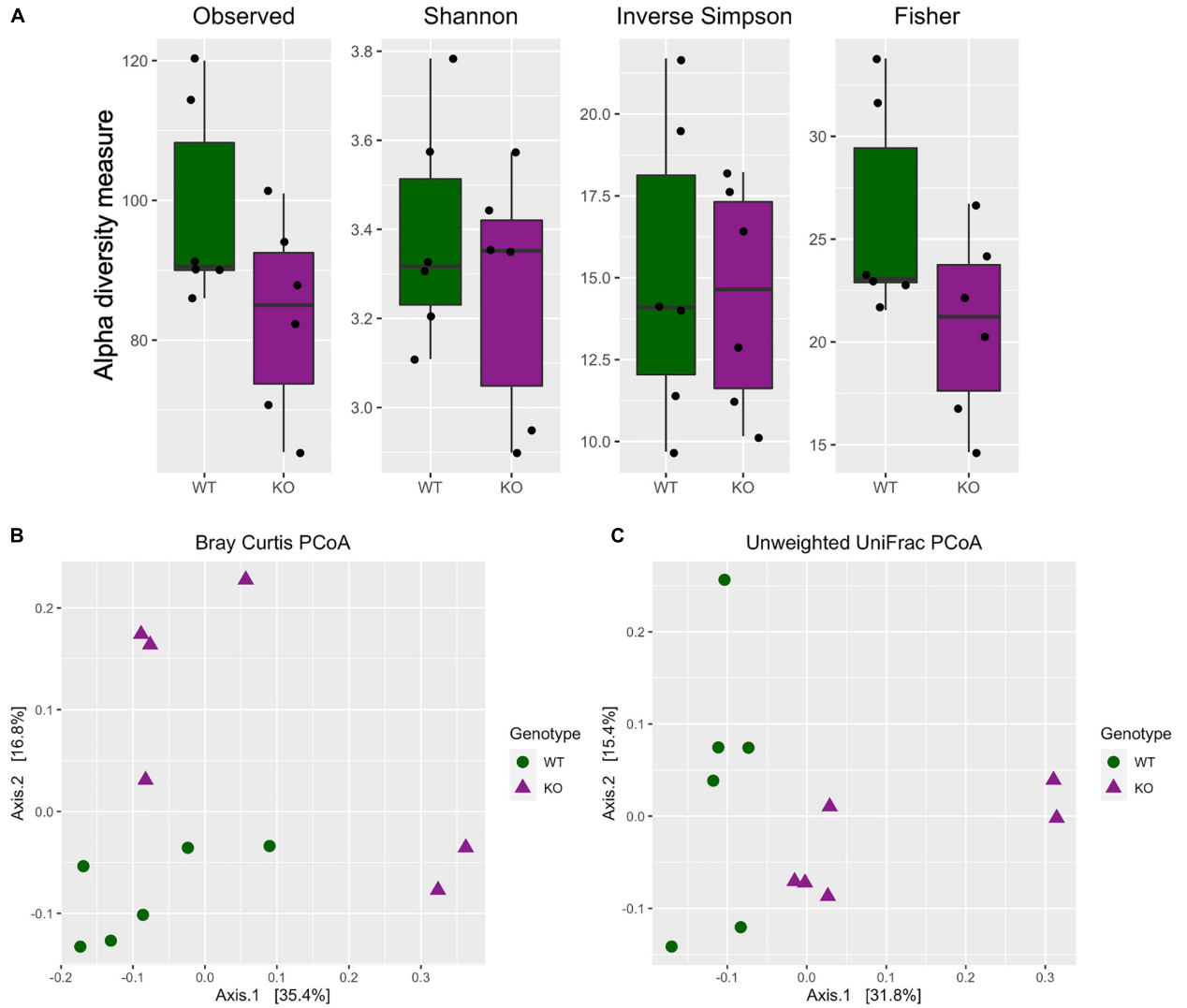

FIGURE 1 | Differential microbiota diversity profiling in mGlu5 KO mice. **(A)** Alpha diversity metrics including Observed, Shannon, Inverse Simpson (InvSimpson), and Fisher indices. There was no difference in all four indices between WT and mGlu5 KO mice (Kruskal–Wallis test). Beta diversity principal coordinate analysis of WT and mGlu5 KO data using **(B)** Bray-Curtis distance and **(C)** unweighted UniFrac distances. The samples were clustered according to the genotype, and there were significant differences between the two genotypes in both Bray-Curtis and unweighted UniFrac distances. Permutation multivariate ANOVA is (n = 6) for both WT and mGlu5 KO mice groups).

(14.6%), *Prevotellaceae* (8.5%), *Bacteroidaceae* (8.5%), and *Erysipelotrichaceae* (2.6%). Finally, a total of 39 genus were detected and 32 of these passed the cut off relative abundance of 0.01% (**Figure 2E**). The most abundant genus was *Unclassified* (from *S24.7* family) with 51.4% of the bacterial population, followed by *Akkermansia* (14.5%), *Bacteroides* (8.5%), and *Prevotella* (8.4%).

Next, we tested differential abundance of bacteria between mGlu5 KO and WT, using analysis of composition of microbiomes (ANCOM), which identified the family *Erysipelotrichaceae* (**Figure 3A**) and the genus *Allobaculum* (**Figure 3B**) as having different levels between genotypes. The relative abundance of these bacteria was identified as being decreased in both family (**Figure 3C**) and genus (**Figure 3D**) in mGlu5 KO mice when compared to WT littermate controls.

Signature of Bacteria Discriminating Both Genotypes (KO and WT)

In order to detect specific ASVs which could contribute to the stratification of samples according to their genotype (as seen above on the PCoA), sPLS-DA was performed (**Figure 4A**). sPLS-DA is a multivariate method performed on the clr-transformed microbiome data to identify microbial drivers discriminating particular phenotype groups. The method identified a signature of bacteria discriminating WT and mGlu5 KO mice, detecting 7 ASVs whose respective contribution is shown on the loading plot

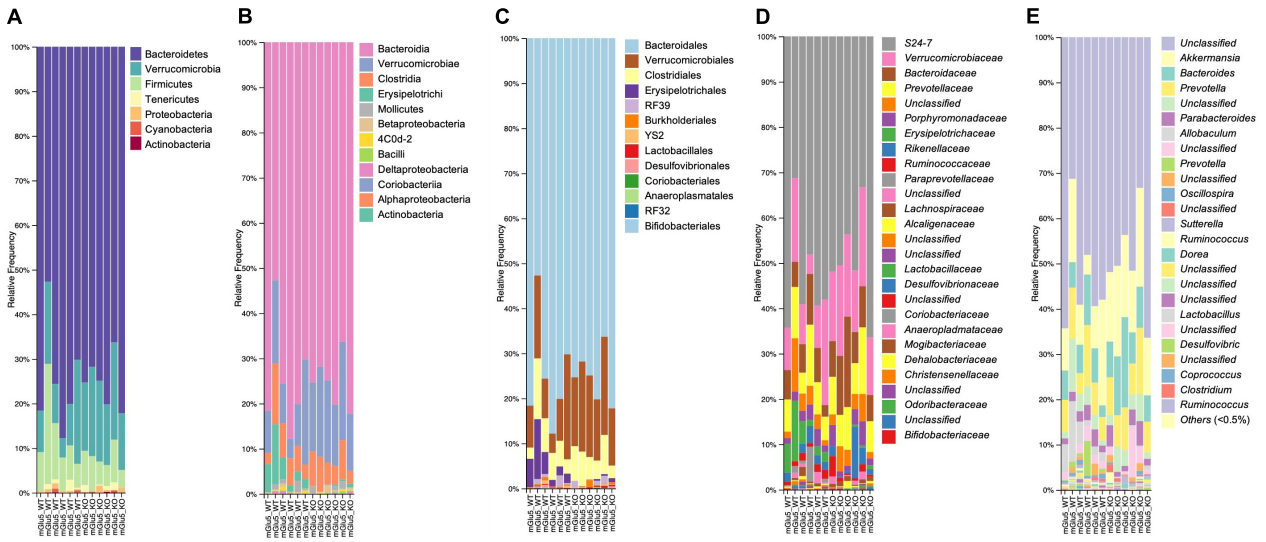

FIGURE 2 | Taxonomic composition distribution histograms of each mGlu5 KO and WT sample. At **(A)** Phylum level, **(B)** class level, **(C)** order level, **(D)** family level, and **(E)** genus level. $n = 6$ for both WT and mGlu5 KO mice groups.

(classification error rate = 0.11, **Figure 4B**). For WT mice, this signature consisted of the Erysipelotrichales, Bacteroidales and Clostridiales order. For mGlu5 KO, this signature consisted of bacteria from the Bacteroidales order.

SparCC-Derived Co-abundance Network Analysis

To obtain a measure of association between ASVs while incorporating their abundance, we used SparCC correlation coefficients that are robust for analyzing compositional microbiome data. A total of 20,301 associations were found, and 78 associations with a p value of less than 0.05 were observed in the network: 46 (59%) of those were positive ($r = -0.6$) and 32 (41%) were negative ($r = -0.6$).

The top 10 keystone taxa (taxa with the most interactions with other taxa) detected in this network are ASV_377, ASV_361, ASV_252, ASV_107, ASV_181, ASV_223, ASV_159, ASV_105, ASV_287 and ASV_99 (none of which are detected by the sPLS-DA). Articulation points are defined as nodes in which when removed with its associated edges, makes the graph disconnected. Some of the articulation points in the network include the ASVs also detected by sPLS-DA: including ASV_557 (genus *Ileibacterium*), ASV_138 (family *Muribaculaceae*), ASV_134 (family *Muribaculaceae)*, and ASV_275 (family *Muribaculaceae)* (**Figure 5**).

Reduced Body Weight in mGlu5 KO Mice Despite No Changes in Food and Water Intake

mGlu5 KO mice presented a reduction in body weight when compared to WT controls (**Figure 6A**; $t = 5.72$, df = 10, $p = 0.0002$). No differences were found in food (**Figure 6B**; $t = 0.04$, df = 11, $p = 0.65$) or water (**Figure 6C**; $t = 1.21$, df = 14, $p = 0.2$) intake between mGlu5 KO mice and WT controls.

Gastrointestinal Parameters

We did not observe any genotype difference in fecal water content (**Figure 7A**; $t = 1.002$, df = 11, $p = 0.33$), fecal output (**Figure 7B**; $t = 0.91$, df = 11, $p = 0.37$), gastrointestinal transit time (**Figure 7C**; $t = 1.06$, df = 11, $p = 0.3$) or gut permeability (**Figure 7D**; $t = 0.72$, df = 11, $p = 0.48$). On the other hand, we observed macroscopic differences between mGlu5 KO and WT mice (**Figure 7E**), including an increase in cecum length (**Figure 7F**; $t = 4.02$, df = 11, $p = 0.002$) and weight (**Figure 7G**); $t = 2.52$, df = 11, $p = 0.02$), together with an increase in colon length (**Figure 7H**; $t = 8.12$, df = 11, $p < 0.0001$), all relative to body weight. We did not find any macroscopic difference before normalizing to the body weight (**Supplementary Figure 2**).

DISCUSSION

This study provides the first evidence that an animal model of SZ replicates the gut dysbiosis observed in patients. In this study, we performed gut microbiome profiling of the mGlu5 KO and WT mice, by 16S rRNA sequencing of fecal samples. We found a significant genotype difference in beta diversity, indicating that mGlu5 KO and WT mice show differences in microbial communities. Specifically, using ANCOM, we uncovered differences in the relative abundance of *Erysipelotrichaceae* family and *Allobaculum* genus. We also identified a signature of bacteria discriminating both genotypes, consisting of the bacterial Order Erysipelotrichales, Bacteroidales, and Clostridiales. This new evidence for gut dysbiosis in mGlu5 KO mice provides unique opportunities to explore new approaches to improve treatment for SZ.

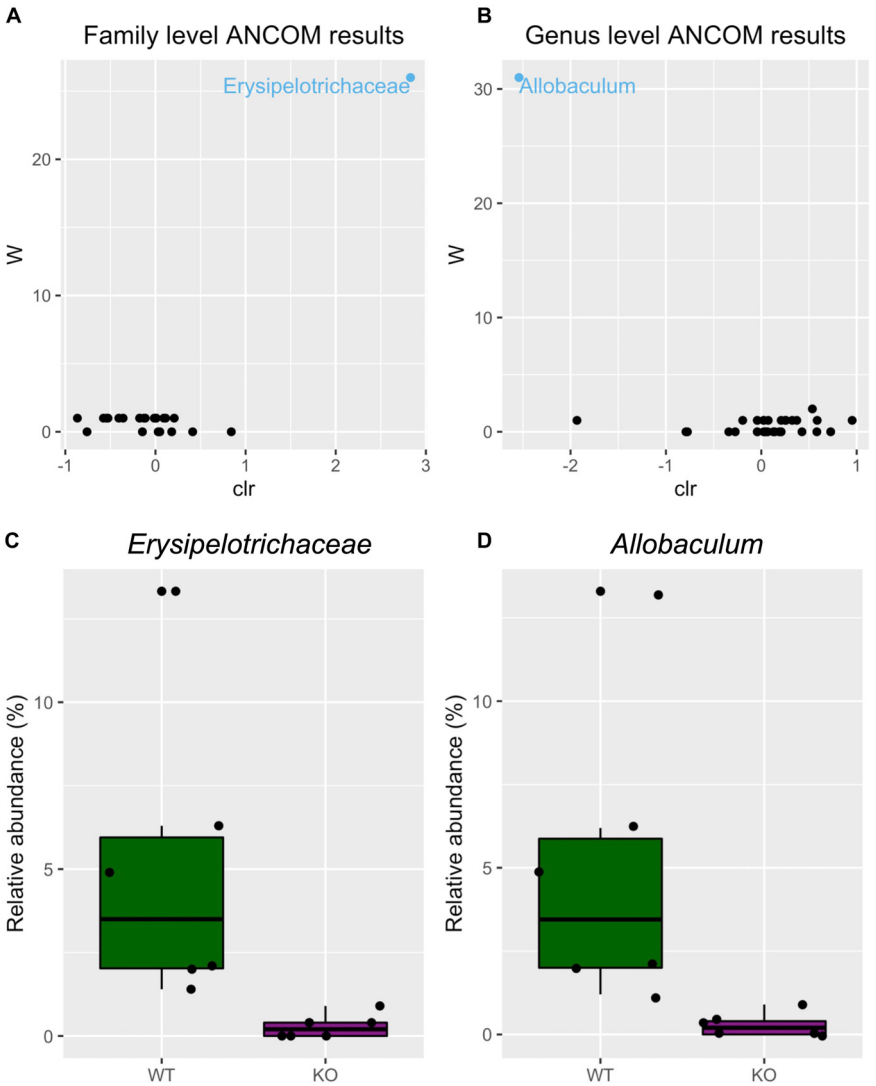

FIGURE 3 | Differential microbiota abundance in mGlu5 KO mice. Volcano plot for the analysis of composition of microbiomes (ANCOM) test for **(A)** family and **(B)** genus level. Only significant bacterial taxa are labeled and colored in blue. Significant taxa are showing high w-stats. Boxplots comparing the relative abundance of the family and genus whose abundance was found to differ significantly between mGlu5 KO and WT **(C)** *Erysipelotrichaceae* and **(D)** *Allobaculum*. The plots show mean ± SEM (n = 6 for both WT and mGlu5 KO mice groups).

There are interesting overlaps between our findings in mice and clinical findings. Similar to our results, previous microbiome profiles of SZ patients have shown no difference between the most abundant phyla Firmicutes and Bacteroidetes (reviewed by Epskamp et al., 2012). Another parallel between our data and clinical SZ findings is that there is no difference in alpha diversity, but a significant difference in beta diversity between SZ patients and controls (Shen et al., 2018; Nguyen et al., 2019). However, it is important to note that while we found a significantly different beta diversity between mGlu5 KO and WT mice, the averaged distance-based coefficient of determination (R^2 of the Bray Curtis and UniFrac results) was approximately 0.2, indicating that only around 20% of the microbiome variation is associated with the genotype. Still, a highly diverse gut microbiome is considered an advantage, being related to a healthy environment and lifestyle, usually associated with low or absent pathogenic bacterial species and also associated with improved cognition (e.g., learning, memory and behavioral flexibility; reviewed by Davidson et al., 2018). Therefore, our finding showing a subtle but significant differential beta diversity in mGlu5 KO mice relative to their WT littermate controls indicates a global differential community composition, and thus gut dysbiosis in this mouse model of SZ.

A highly diverse, as well as a balanced composition, of the microbial community is crucial to the homeostasis of the gut microbiome. Gut bacteria regulate basic processes such as digestion, maintenance of intestinal epithelium integrity and, importantly, commensal bacteria promote the first immune response against pathogenic bacteria (Rinninella et al., 2019).

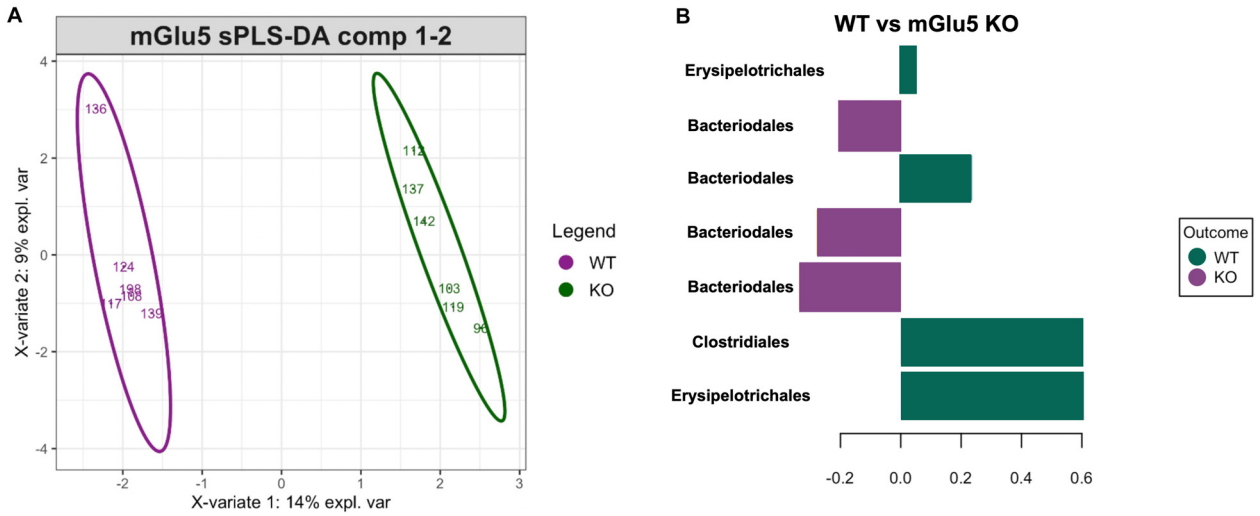

FIGURE 4 | Identification of a bacterial signature discriminating the genotypes with sPLS-DA. **(A)** Sample plots with 0.95 confidence ellipse plots for WT and mGlu5 KO show a strong discrimination between genotypes. The top 7 resulting bacterial signature contributing to component 1 is displayed in **(B)**. The length of the bars indicates the importance of each amplicon sequence variants (ASVs) (at the Order taxonomic rank) in the signature (from bottom to top: decreasing importance) with color indicating the phenotype group with maximum median abundance (n = 6 for both WT and mGlu5 KO mice groups, classification error rate = 0.11).

Furthermore, gut bacterial diversity and community composition determine the abundance of SCFAs, end-products of microbial fermentation which are vital for gut health (Campbell et al., 2016). One limitation of our study is that we had a relatively low sequencing depth at the alpha rarefaction curve, which could have influenced the alpha diversity analysis specifically. However, when we conducted an analysis investigating the microbial diversity for each sample at various sampling depths, we observed that in all cases the alpha diversity between the two genotypes was not significantly different (**Supplementary Table 2**).

When comparing humans and mice, there are extensive similarities in anatomy, physiology and genetics which have allowed numerous inferences about human health and pathologies. In gut microbiota research, mouse models have been increasingly used to study the role of the gut microbiota and its association with diseases (Nguyen et al., 2015). However, there are intrinsic differences between these two mammalian systems that make absolute comparisons challenging, particularly at the species level (Hugenholtz and de Vos, 2018). When care is taken in drawing direct parallels between murine and human gut, with regard to microbiota composition, mouse models provide valuable tools to study microbiota imbalance or dysfunction. Thus, our main goal with this study was to establish whether our mouse model of SZ provided an appropriate tool for preclinical studies of SZ. Our findings in the present study demonstrate that these mGlu5 KO mice may model the gut microbiota differential community composition observed in patients.

Using ANCOM we observed a decrease in the relative abundance of the family *Erysipelotrichaceae* in mGlu5 KO mice while the order Erysipelotrichales was one of the associated bacteria-signature discriminating mGlu5 KO and WT mice. In contrast, the same bacterial family was increased in adult mice born from a maternal immune activation (MIA) animal model of gestational influenza virus infection (Saunders et al., 2020) and a species from this family, *Catenibacterium mitsuokai*, was found relatively more abundant in SZ oral samples than in controls (Castro-Nallar et al., 2015). ANCOM analysis also indicated a decrease in the genus *Allobaculum* in mGlu5 KO mice when compared to WT. This genus is a member of the family *Erysipelotrichaceae* and is associated with a healthy microbiome, suggested to have beneficial gut effects related to mucus properties and formation (Everard et al., 2014). A decrease in the abundance of *Allobaculum* has been observed in the APP/PS1 mouse model of Alzheimer's disease (Harach et al., 2017) and absent in restrained mice as a depression-like animal model, while high in the control group (Koo et al., 2010). Importantly, the treatment with the antipsychotic risperidone in mice was shown to increase the abundance of *Allobaculum* (Bahr et al., 2015).

The order Bacteriodales was one of our signature bacteria responsible for discriminating our genotypes (mGlu5 KO versus WT mice) and it was previously associated with individuals with ultra-high risk for psychosis, when compared to healthy controls (He et al., 2018). In addition, the genus of the same taxa *Bacteroides*, was associated with SZ in a recent gene set enrichment analysis (GSEA) study that used published psychiatric disorder GWAS data, as well as GWAS of gut microbiota summary data (Cheng et al., 2020). Clostridiales by its turn, another signature bacteria responsible to discriminate our SZ mouse model from control WT mice in our study, was also already associated with ultra-high risk for psychosis individuals when compared to healthy controls (He et al., 2018). Families from this order were demonstrated to be decreased in SZ patients, suggesting an impairment in the maintenance of gut health (Zheng et al., 2019). Other studies also demonstrated an association between lower levels of the Clostridiales order

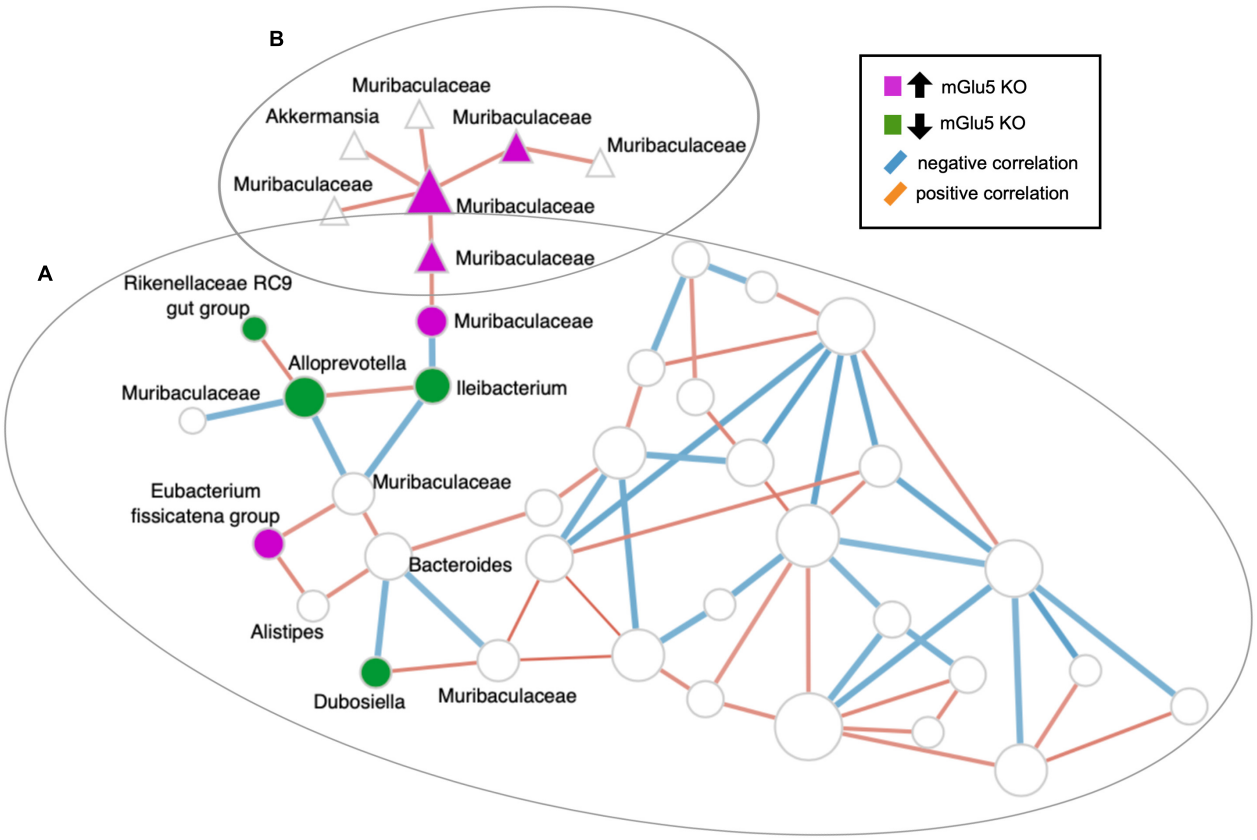

FIGURE 5 | SparCC-derived co-abundance network analysis. Nodes belonging to the same clusters calculated using the walktrap algorithm are depicted by their shapes (all nodes with circles belong to cluster **(A)**, all nodes with triangles belong to cluster **(B)**). Nodes that were detected by sPLS-DA and their first neighbors were labeled. Nodes colored in magenta are amplicon sequence variants (ASVs) detected by sPLS-DA to be increased in KO mice, while the nodes colored in dark green are ASVs detected by sPLS-DA to be decreased in KO mice. ASVs are shown at the Genus level, in the case where Genus is not assigned, the next highest taxonomy level is shown. The edges (lines connecting two circles) are colored according to their correlation: blue for negative correlation, red for positive correlation. Isolated nodes are omitted from the network.

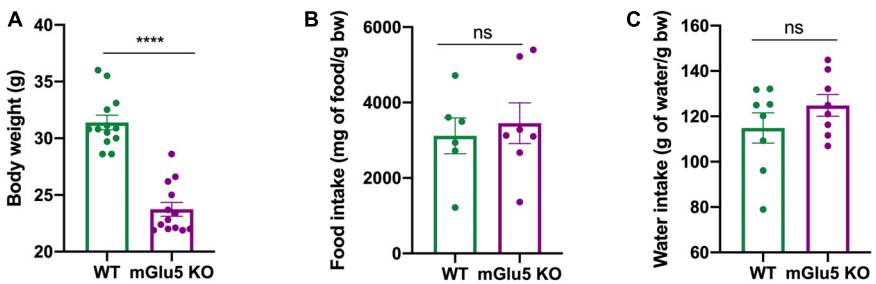

FIGURE 6 | Reduced body weight in mGlu5 KO mice despite no changes in food and water intake. **(A)** Decreased body weight in mGlu5 KO mice compared to WT at 12 weeks of age, unpaired *t*-test. **(B)** Cumulative food intake (represented as mg of food per g of body weight) and **(C)** cumulative water intake (represented as g of water per g of body weight), both over 4 weeks between mGlu5 KO mice and WT controls, unpaired *t*-test. The plots show mean ± SEM (n = 6–8 for both WT and mGlu5 KO mice groups). ***$p < 0.001$.

in SZ patients (Shen et al., 2018). Furthermore, a negative correlation between *Clostridium_sensu_stricto_1* and the positive and negative syndrome scale (PANSS) score, which is a medical scale used for measuring symptom severity of patients with SZ, was recently reported, suggesting that changes in intestinal microbiota may modulate the prognosis of the disorder (Pan et al., 2020). We also performed a co-occurrence network analysis using SparCC to identify associations between the microbes. We observed that the main hubs (or keystone taxa) of the network are not microbes detected by sPLS-DA. The

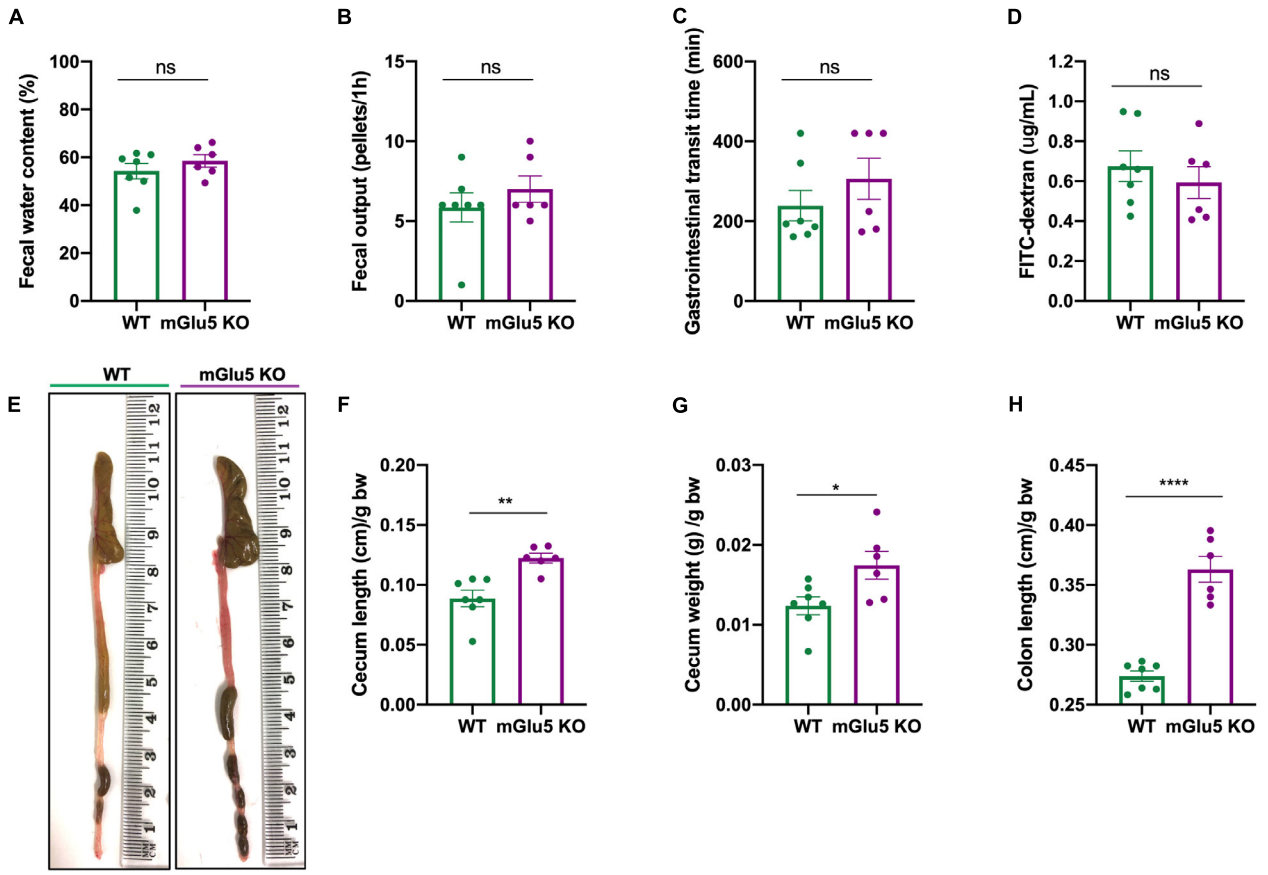

FIGURE 7 | Gastrointestinal parameters of mGlu5 KO mice. **(A)** Fecal water content as a percentage of difference between the initial total feces weight and the dry weight. **(B)** Fecal output, counting the number of fecal pellets expelled during 1 h. **(C)** Gastrointestinal transit time, using the non- absorbable red dye Carmine red and recording first appearance in the feces. **(D)** Gut permeability measured by 4 kDa fluorescein isothiocyanate (FITC)-dextran assay. **(E)** Macroscopic evaluation of intestine. **(F)** Cecum length, **(G)** cecum weight, and **(H)** colon length normalized by body weight, unpaired *t*-test. The plots show mean ± SEM (n = 6–7 for both WT and mGlu5 KO mice groups). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and ns $p > 0.05$.

microbes identified by sPLS-DA are clustered together and their interactions are mainly local (within their own neighborhood). Some of these sPLS-DA identified microbes are also articulation points, in which they bridge the connection between two separate neighborhoods. However, we acknowledge that this approach may be insufficient in inferring microbial community structure (Hirano and Takemoto, 2019).

We have therefore identified interesting overlaps between our present findings and previous clinical SZ literature, with promising translational bacterial targets. However, it is important to note that, regarding specific taxa abundance and enrichment, the discrepancies between clinical studies are even more prominent, mainly due to methodological differences and inherent limitation of the clinical heterogeneity of SZ (Macedo e Cordeiro et al., 2020). Therefore, a simple gut microbiome signature for SZ patients is still not determined. It is essential in clinical studies to rigorously control for treatment, diet, physical activity and previous prebiotic/probiotic/antibiotic use, among other confounders; however, this is not always possible. Nevertheless, the fully controlled environment (as well as genetic control) that animal studies provide can overcome these limitations, and preclinical studies that replicate a general dysbiosis observed in patients, including this mGlu5 KO mouse model, represent a useful tool for the study of the gut microbiome in psychiatric disorders.

A few studies with animal models of relevance to SZ have previously suggested a promising potential of a microbial community role associated to these preclinical models. Sub-chronic N-methyl-D-aspartate receptor (NMDAR) antagonist phencyclidine (PCP) administration in male Lister Hooded rats resulted in cognitive impairment and differential beta diversity (Pyndt Jørgensen et al., 2015). A correlation between gut microbiota and memory performance was also demonstrated, in addition to the administration of antibiotic being able to reverse the cognitive deficits observed in sub-chronic PCP treated rats (Pyndt Jørgensen et al., 2015). However, this last result should be carefully interpreted since they did not investigate the direct central effect that ampicillin is able to perform, and thus this antibiotic may have acted directly on the brain rather than via the gut (Nau et al., 2010). Similarly, in an MIA model of maternal

influenza viral infection and associated neurodevelopmental disorder, cognitive impairments were observed in the offspring and antibiotic administration during their prepuberal period was able to prevent that outcome (Saunders et al., 2020). Additionally, the same study showed that adult MIA offspring displayed altered gut microbiota, with differential relative abundance between components of the gut microbiota, including *Ruminococcaceae*. Likewise, in Wistar rats, an animal model combining MIA with polyI:C and adolescent cannabinoid exposure demonstrated a sex-specific effect, with adolescent female offspring exhibiting decreased fecal levels of *Bifidobacterium longum,* measured using qPCR (Katz-Barber et al., 2020). While very promising, these aforementioned studies should be interpreted with caution, since recapitulating the complexity of SZ in an animal model is still a challenge, and each study has its own limitations. In particular, the PCP model lacks construct validity (Sams-Dodd, 1999) and MIA animal models are associated with a broad range of disorders, including ASD, and thus MIA may be considered to be a non-specific primer of multiple neurodevelopmental disorders (Estes and McAllister, 2016).

The genetics of SZ is complex and heterogeneous and thus genome-wide association studies (GWAS) do not identify single mutations strongly associated with this psychiatric disorder. However, mGlu5 has been implicated in SZ pathogenesis (Devon et al., 2001; Gupta et al., 2005), along with many related proteins associated with synapse development and function. Decreased mGlu5 mRNA and protein levels in SZ patients have been reported in postmortem studies (Volk et al., 2010; Matosin et al., 2015). The mGlu5 KO mice demonstrate impaired prepulse inhibition (PPI) (Brody and Geyer, 2004; Burrows et al., 2015), baseline hyperactivity and a hypersensitivity to MK-801-induced hyperlocomotion (Lipina et al., 2007; Gray et al., 2009), impaired circadian process (Aguilar et al., 2020), clinically relevant cognitive deficits on touchscreen tasks (Zeleznikow-Johnston et al., 2018) and thus excellent face validity as a preclinical model of SZ. Lastly, chronic treatment with clozapine is able to reverse SZ-related behaviors (Gray et al., 2009), demonstrating strong predictive validity. Taken together, these studies indicate that mGlu5 KO mouse model has great utility for the study of SZ, including the altered microbiome associated with gut dysbiosis.

Recently, a study focused on the association between depressive behavior and the mGlu5 receptor reported 16S rRNA sequencing in feces from another line of mGlu5 KO mice, concluding that there were no gut microbiota changes between genotypes (Cai et al., 2020). Discrepancies between this study and ours are likely to result from multiple factors, including differences in the generation and genetic background of mGlu5 KO mice. In their study, Cai and colleagues generated mGlu5 KO mice by crossing $Grm5^{flox/flox}$ mice with B6.C-Tg (CMV-cre) mice but did not explore SZ-like endophenotypes. Our Grm5tm1Rod line of mice have a constitutive null mutation in the mGlu5 gene, with strong face and predictive validity as a preclinical model of SZ (Hannan et al., 2001; Gray et al., 2009; Burrows et al., 2015). In addition, Cai and collaborators have not extensively analyzed their microbiome data. In particular, similar to their study, we didn't observe any difference between alpha diversity, but when we analyzed other levels of complexity, such as beta diversity and sPLS-DA (low classification error rate), we discovered a genotype effect. Therefore, following thorough and exhaustive microbiome profiling, our present findings are the first to demonstrate gut dysbiosis in the mGlu5 KO mouse model of SZ.

Consistent with previous findings (Bradbury et al., 2005), we also observed a reduced body weight in mGlu5 KO mice when compared to WT controls, despite no changes in food and water intake between groups. In fact, mGlu5 has been suggested to be a mediator of energy balance in rodents by decreasing caloric efficiency, suggesting increased energy expenditure in mGlu5 KO mice (Bradbury et al., 2005). Corroborating these findings, it was also demonstrated that activation of lateral hypothalamic mGlu5 receptors elicits feeding in rats (Ploj et al., 2010; Charles et al., 2014) and mGlu5 KO mice are resistant to diet-induced obesity (Bradbury et al., 2005). Interestingly, the abundance of the genus *Allobaculum,* which we have found decreased in mGlu5 KO mice, has been correlated with body weight and dietary-induced inflammation markers, including leptin and IL-22 (Ravussin et al., 2012; Zenewicz et al., 2013; Everard et al., 2014). Considering the highly explored and relevant role of gut microbiota composition in the pathogenesis of obesity (Leocádio et al., 2020), our novel findings showing a differential gut microbiome profile between mGlu5 KO and WT mice may have relevance to the study of metabolic and eating disorders, including obesity.

Glutamate is a central and peripheric modulator, with glutamatergic dysfunction associated with both central nervous system (CNS) disorders and GI diseases, which in many cases display intercorrelated co-morbidities (e. g. inflammatory bowel disease (IBD) and depression) (Keefer and Kane, 2017; Baj et al., 2019). Not surprisingly, SZ is also associated with GI issues, such as gut inflammation and gut cellular barrier defects (Severance et al., 2015). In fact, the glutamatergic system is directly implicated in GI modulation, with the mGlu5 receptor being also expressed in the GI tract (Ferrigno et al., 2017) and involved with the peripheral excitatory modulation of vagal afferent mechanosensitivity (Slattery et al., 2006). Interestingly, antipsychotics, the main treatment for SZ, induce weight gain, constipation and metabolic syndrome (Kanji et al., 2018; Zeng et al., 2020). It is possible that the negative side effects of antipsychotics may be at least partly related to an antibiotic-like side effect on gut microbiota, by altering its composition and decreasing diversity (reviewed in (Dinan and Cryan, 2018; Skonieczna-Żydecka et al., 2019)). Thus, microbiota interventions adjunctively with antipsychotics are promising to alleviate adverse side effects and mGlu5 KO mice may provide a useful preclinical tool to investigate these and other aspects of SZ pathogenesis and treatment.

Considering that the interactions between microbiota composition and gut status are bidirectional, with the bacteria population playing an important role in gut function and the gut function affecting the diversity of the microbiome in the GI tract (Barbara et al., 2005), we evaluated GI health parameters.

We didn't see any difference in fecal water content, fecal output or gastrointestinal transit time, which have been interrelated with gut microbiota composition (Kashyap et al., 2013; Vandeputte et al., 2016). Similarly, we didn't see a difference in gut permeability, which has been linked to dysbiosis and increased inflammation (Chakaroun et al., 2020). One possible explanation is that, due to the fact that only around 20% of the microbiome variation revealed by beta diversity is associated to the genotype, dysbiosis can potentially not be strong enough to modulate or be reflected in the gastrointestinal parameters analyzed. On the other hand, after normalization by body weight we observed macroscopic differences between mGlu5 KO and WT mice, including an increase in cecum length and weight, together with an increased colon length. Since colon length can be considered a marker of colonic inflammation (Chassaing et al., 2014) and cecum measurements also associates with gut microbiota composition (Ge et al., 2017) further study is needed to explore whether mGlu5 KO mice present gastrointestinal dysfunction associated with inflammation. Therefore, clarifying the extent of the gut dysbiosis in this mGlu5 KO mouse model and establishing whether gut dysbiosis is causally associated with GI dysfunction, would be of great interest.

CONCLUSION

In conclusion, the microbiome phenotype observed in mGlu5 KO mice is to some extent in line with reports of gut dysbiosis in SZ patients, and thus this animal model provides a novel tool to explore the mechanistic understanding of how and when dysbiosis arises in SZ. Furthermore, our present findings can be used to explore the link between dysbiosis and SZ symptoms (i.e., behavioral impairments) as well as the potential utility of gut microbiome restoration as a therapeutic approach (e.g., fecal matter transplantation, probiotics and prebiotics), while clarifying the extent of microbiota–gut–brain axis dysfunction in this model. Moreover, considering that mGlu5 KO mice also present deficits in basic discrimination learning and cognitive flexibility (Zeleznikow-Johnston et al., 2018), this mouse model can be also used as a tool for the investigation of the role of the gut microbiome in generalized cognitive impairments. Thus, our present findings may also inform other cognitive disorders where mGlu5 receptors play a role and the microbiome association is still not well understood, such as addiction (Terbeck et al., 2015; Meckel and Kiraly, 2019).

Our results provide the first evidence that a genetic animal model of SZ, exhibiting both face and predictive validity, at least partly replicates the gut dysbiosis observed in patients. These new findings of gut dysbiosis in the mGlu5 KO mice provide opportunities to explore novel approaches focusing on the microbiota–gut–brain axis in SZ. We therefore propose that this preclinical model of SZ can be used as a tool to investigate how gut dysbiosis may contribute to SZ pathogenesis, via the microbiota–gut–brain axis. Our new findings may also inform the development of novel therapeutic approaches for SZ, a devastating disorder for which new treatments are urgently needed.

DATA AVAILABILITY STATEMENT

The datasets and metadata related to this study have been deposited in the NCBI Sequence Read Archive under BioProject number PRJNA659149. Furthermore, the R code for the analysis has been uploaded to the following site: https://github.com/gkong1/mGLU5KO_microbiome_analysis.

ETHICS STATEMENT

The animal study was reviewed and approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

CG was involved in the experimental design, data collection, data analysis, and manuscript writing. GK was involved in microbiome data analysis. VU, AZ-J, EB, and TR were involved in the data collection, analysis, and editing of the manuscript. AH was involved in the experimental design, project management and funding, data analysis, and drafting of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

CG is a recipient of Melbourne Early Career Researcher Award. EB is an NHMRC-ARC Dementia Research Development Fellow. TR is an NHMRC Dementia Fellow. AH is an NHMRC Principal Research Fellow and is also supported by NHMRC Project Grants, an ARC Discovery Project and the DHB Foundation, Equity Trustees.

ACKNOWLEDGMENTS

We thank Jamie Liew for helping with sample collection and Shanshan Li for laboratory management.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.582320/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 19 November 2020
doi: 10.3389/fcell.2020.562812

The Gut Microbiota, Kynurenine Pathway, and Immune System Interaction in the Development of Brain Cancer

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: 16 May 2020
Accepted: 26 October 2020
Published: 19 November 2020

Citation:
Dehhaghi M, Kazemi Shariat Panahi H, Heng B and Guillemin GJ (2020) The Gut Microbiota, Kynurenine Pathway, and Immune System Interaction in the Development of Brain Cancer. Front. Cell Dev. Biol. 8:562812. doi: 10.3389/fcell.2020.562812

Human gut microbiota contains a large, complex, dynamic microbial community of approximately 10^{14} microbes from more than 1,000 microbial species, i.e., equivalent to 4×10^6 genes. Numerous evidence links gut microbiota with human health and diseases. Importantly, gut microbiota is involved in the development and function of the brain through a bidirectional pathway termed as the gut-brain axis. Interaction between gut microbiota and immune responses can modulate the development of neuroinflammation and cancer diseases in the brain. With respect of brain cancer, gut microbiota could modify the levels of antioxidants, amyloid protein and lipopolysaccharides, arginase 1, arginine, cytochrome C, granulocyte–macrophage colony-stimulating factor signaling (GM-CSF), IL-4, IL-6, IL-13, IL-17A, interferon gamma (IFN-γ), reactive oxygen species (ROS), reactive nitrogen species (e.g., nitric oxide and peroxynitrite), short-chain fatty acids (SCFAs), tryptophan, and tumor necrosis factor-β (TGF-β). Through these modifications, gut microbiota can modulate apoptosis, the aryl hydrocarbon receptor (AhR), autophagy, caspases activation, DNA integrity, microglia dysbiosis, mitochondria permeability, T-cell proliferation and functions, the signal transducer and activator of transcription (STAT) pathways, and tumor cell proliferation and metastasis. The outcome of such interventions could be either oncolytic or oncogenic. This review scrutinizes the oncogenic and oncolytic effects of gut microbiota by classifying the modification mechanisms into (i) amino acid deprivation (arginine and tryptophan); (ii) kynurenine pathway; (iii) microglia dysbiosis; and (iv) myeloid-derived suppressor cells (MDSCs). By delineating the complexity of the gut-microbiota-brain-cancer axis, this review aims to help the research on the development of novel therapeutic strategies that may aid the efficient eradication of brain cancers.

Keywords: indoleamine 2,3-dioxygenase-1, anti-tumor T-cells, tryptophan, gut microbiota, myeloid-derived suppressor cells, kynurenine pathway, glioblastoma

INTRODUCTION

The incidence of primary brain cancer is estimated to be 7.2–12.5 per 100 million persons per year, accounting for up to 2% and 23% of all adults and childhood cancers, respectively (Wrensch et al., 2002; Marie and Shinjo, 2011). Among the primary brain tumors, astrocytoma, originates from glial cells is the most frequent brain tumor. The most malignant form of astrocytoma is glioblastoma, which has an incidence rate of 3 per 100,000 persons (Ostrom et al., 2017). Currently, the main causes of many brain tumors remain unknown. However, it has been suggested that some internal factors (i.e., genetic elements such as *POT1*) and external factors (i.e., environmental factors such as ionizing irradiation) could increase the risk of brain tumors (Picano et al., 2012; Robles-Espinoza et al., 2014).

It is well-known that some microorganisms have oncogenic or oncolytic activity on tumor cells. For example, it is estimated that up to 20% of all cancers are triggered by infectious agents (e.g., human papillomaviruses, *Helicobacter pylori*, and hepatitis B and C viruses) (De Martel et al., 2012). Interestingly, healthy individuals and cancer patients have different microbial flora in terms of population and diversity (Xuan et al., 2014). The influence of gut microbiota in various cancers has been extensively studied (Loo et al., 2017; Mehrian-Shai et al., 2019; Wong et al., 2019). However, its possible association with brain cancer is a new topic. Understanding the mechanisms of involvement of the gut microbiota-brain axis in the development or suppression of brain tumor could establish a new insight for the generation of novel anti-tumor therapeutic interventions. The gut-brain axis represents a complex multidirectional network between the gastrointestinal (GI) tract microbiota, the enteric nervous system, and the brain that influences immune responses, inflammation processes, and metabolic functions (Fung et al., 2017; Dehhaghi et al., 2019a).

The kynurenine pathway is the main route of tryptophan metabolism that results in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) and various neuroactive intermediates (Guillemin et al., 2007; Dehhaghi et al., 2019a). In last decades, the involvement of the kynurenine pathway in brain diseases particularly brain tumors has gained more attention. Tryptophan is also catabolized through serotonin pathway that leads to biosynthesis of neuroactive metabolites such as serotonin and melatonin. It is important to note that gut microorganisms utilize tryptophan as a substrate to produce indoles indole derivatives, which are key molecules involved in signaling pathways between GI tract and immune system (Agus et al., 2018). Dysregulation of the kynurenine pathway could contribute to cancer development by disrupting the antitumoral immune response (Adams et al., 2012; Platten et al., 2019). The expression of indoleamine 2,3-dioxygenase (IDO1) and tryptophan 2,3-dioxygenase (TDO), two main enzymes contributing in tryptophan degradation, has been linked to various cancers such as melanoma, colon cancer, gynecological malignancies, lung cancer, gliomas, and bladder cancer (Théate et al., 2015; Amobi et al., 2017; Platten et al., 2019). In addition, involvement of the kynurenine pathway metabolites such as kynurenine, quinolinic acid, and 3-hydroxyanthranilic acid in cancer progression has been previously investigated (Adams et al., 2012). To the best of our knowledge, this review is the first study that comprehensively discusses the possible involvement of gut microbiota in brain cancers. The speculative mechanisms for this gut-microbiota-brain-cancer axis have been scrutinized in four sections: (i) through the kynurenine pathway; (ii) through mediation of types 1 and 2 T helper cells and the subsequent modulation of microglia (independent of the kynurenine pathway); (iii) myeloid-derived suppressor cells (MDSCs); and (iv) amino acid deprivation (i.e., arginine and tryptophan). By providing state-of-the-art information on the gut-microbiota-brain-cancer axis, this review aims to help cancer researchers and clinicians with the development of novel anti-tumor therapeutic strategies.

THE GUT MICROBIOTA-BRAIN AXIS

Microorganisms occur ubiquitously and have abilities to metabolize a diverse range of metabolites ranging from enzymes (Hamedi et al., 2015; Mohammadipanah et al., 2015), therapeutic lead compounds (Mohammadipanah et al., 2016; Sajedi et al., 2018), and antioxidants (Dehhaghi et al., 2018b, 2019a,c, 2020) to hydrocarbon-rich compounds, i.e., ethanol (Dehhaghi et al., 2019a; Kazemi Shariat Panahi et al., 2019b), butanol (Dehhaghi et al., 2019b), and methane (Dehhaghi et al., 2019b; Tabatabaei et al., 2019).

Human gut is one of the most dynamic niches. It contains a large and complex community of microorganisms with approximately 10^4 microbial species, i.e., equivalent to 4×10^6 genes. Interestingly, gut bacterial density has been found to be 10^{12}/mL, the largest microbial density in any given ecosystem (Bhattacharjee and Lukiw, 2013; Dehhaghi et al., 2018a). Firmicutes (∼51%) and Bacteroidetes (∼48%) are the top two most abundant bacterial phyla in the human gut in respect of population, followed by other bacterial phyla including Actinobacteria, Cyanobacteria, Fusobacteria, Proteobacteria, and Verrucomicrobia (Eckburg et al., 2005; Sekirov et al., 2010; Dehhaghi et al., 2019a). These microorganisms could have a profound role in human health and diseases (Dehhaghi et al., 2018a).

Recently, it has been suggested that human gut microbiota can modulate the development and function of the central nervous system (CNS) through a bio-directional pathway termed as the gut-brain axis (Barbara et al., 2007; Foster and Neufeld, 2013; Dehhaghi et al., 2018a). The regulation of brain function occurs through modulating key processes such as neuroinflammation, neurogenesis, and neurotransmission. Microorganisms that reside in the GI tract can influence the brain activities through synthesizing neurotransmitters [i.e., dopamine, γ-amino butyric acid (GABA)] and short chain fatty acids (SCFAs), or through modulating immune responses and amino acid metabolism (Sherwin et al., 2016; Dehhaghi et al., 2019a). Many *in vivo* studies have shown that germ-free mice suffered from upregulation of genes that are associated to plasticity, steroid hormone metabolism, and synaptic long-term potentiation in the

hippocampus, compared to normal mice (Buffington et al., 2016; Dehhaghi et al., 2018a; Spichak et al., 2018).

In CNS, for example, vagal afferents are responsible for transferring sensory messages (i.e., gut distension, food availability, and motor activity) to the nucleus of the solitary tract located in the brain stem (Furness et al., 2014). Neuronal inputs are subsequently sent to the higher parts of the CNS or are involved in long vago-vagal reflexes (Mulak and Bonaz, 2004). Afferent fibers in the vagus nerve direct the signals from the gut to secondary afferent neurons located in the dorsal horn. Secondary afferent neurons project to the CNS with the aid of spinothalamic pathways, which are considered to be the major pain signaling routes in the gut-brain axis (Mulak and Bonaz, 2004; Furness et al., 2014). In addition, the enteric nervous system is responsible for receiving and transmitting signals to and from the autonomic nervous system, providing a critical role in the gut-brain axis communication (Furness, 2012). A growing body of evidence has highlighted the obvious impact of the gut microbial community on the gut-brain axis functions (Quigley, 2017; Dehhaghi et al., 2018a, 2019a).

It is important to note that gut microorganisms can directly stimulate the afferent sensory neurons. Microbial SCFAs have a profound influence on enteroendocrine cells for producing various enteric neuropeptides. These can pass through the lamina propria and reach the blood stream and relevant receptors to strongly impact the extrinsic vagal innervation or enteric nervous system neurons. Bacterial circulating SCFAs, such as butyrate and propionate can bind to monocarboxylate transporters, which are extensively present at the blood-brain-barrier (BBB) and enter the CNS (Maurer et al., 2004). Monocarboxylate transporters are also expressed on the surface of neuronal and glial cells, providing a mechanism through which these compounds can be taken up and utilized as the main source of cellular energy particularly in early stages of brain development (Pellerin, 2005; Burokas et al., 2015).

It should be noted that the diversity, population, and metabolites of gut microbiota may control the state and level of both local and systemic immunity. The modulation of the immune system may be through the kynurenine pathway or through a direct effect on immune cells. For example, a bidirectional communication exists between gut microbiota and neuroendocrine system through the hypothalamic-pituitary-adrenal (HPA) axis (Farzi et al., 2018). HPA axis is a significant neuroendocrine system that controls body to ensure adequate responses to physical and psychological stressors (Smith and Vale, 2006). It has been observed that the existence of the gut microbiota in early stages of life could influence neuroendocrine responses to stress (Dinan and Cryan, 2012; O'Mahony et al., 2017). Furthermore, some disorders related to gut microbiota such as irritable bowel syndrome has a potential link with neuroendocrine system disorder such as depression. Interestingly, HPA axis is reportedly increased in both mentioned disorders (Videlock et al., 2016; Juruena et al., 2018). A reverse relationship is also true through which, for example, irritable bowel syndrome is resulted following the generation of depression due to chronic or early life stresses (Whitehead et al., 1992; Liu et al., 2017; Farzi et al., 2018). More specifically, the activation of HPA axis can increase gastrointestinal permeability and the gut microbiota composition (Heim et al., 2000; De Punder and Pruimboom, 2015). However, to this date, there is no reports on the interaction of gut microbiota in the development of brain cancer through HPA axis. In respect to brain cancer, gut-microbiota may influence the tumor microenvironment by controlling T-cell expansion and activation, microglia, cytokines production, arginine and tryptophan availability, kynurenine pathway, and ROS and antioxidants generation (**Table 1**).

AMINO ACID DEPRIVATION

Gut microbiota can decrease the availability and metabolism of some dietary amino acids such as tryptophan (see Section "Tryptophan") and arginine by utilizing them for the production of microbial protein and various metabolites (Mardinoglu et al., 2015; Dehhaghi et al., 2019a). The depletion of these amino acids could influence the progression and severity of tumor cells, including GBM.

Arginine

Arginine is a semi-essential amino acid for humans that can be provided through diet, endogenous synthesis, and protein turnover. The human body uses arginine to produce several metabolites, some of which may affect tumors. Some pro-tumor arginine-derived metabolites in the body include polyamines and nitric oxide. Through depletion of dietary arginine, gut microbiota may decrease the metabolism of arginine-derived pro-cancer metabolites by decreasing the arginine flux in body. However, gut microbiota could also produce some arginine-derived pro-cancer metabolites during the assimilation of dietary arginine. For example, polyamines and nitric oxide are two arginine-derived metabolites that are produced by gut microbiota (Dai et al., 2015; Kao et al., 2015). The produced polyamines, then, could translocate into the brain by blood circulatory system. Following passing blood-brain-barrier, the microbial originated polyamines may induce the proliferation and metastasis of tumor cells through upregulating the expressions of ornithine decarboxylase, spermidine/spermine acetyltransferase, and Akt1 (Dai et al., 2017).

The impact of nitric oxide on cancer cells is controversial and significantly depends on its concentration, exposure time, cell type, and microenvironment. High levels of nitric oxide could suppress anti-tumor T-cell activity through enriching MDSCs (see Section "Myeloid-Derived Suppressor Cells"). Moreover, aberrant production of nitric oxide by gut microbiota could increase the flux of peroxynitrite, following its reaction with superoxide radicals. Peroxynitrite is one of the key compounds that promotes MDSC suppressive activity on T-cell function (see Section "Myeloid-Derived Suppressor Cells"). Alternatively, nitric oxide interferes with T-cell function through the inhibition of MHC class II transcription (Rivoltini et al., 2002), the inhibition of the JAK3-STAT5 signaling pathway, and the induction of T-cell apoptosis (Bingisser et al., 1998; Harari and Liao, 2004). Furthermore, high levels of nitric oxide

TABLE 1 | Some important factors in cancer that are affected by gut microbiota.

Factor	A[1]	Presence of gut microbiota			References
		Genus/Strain	**B[2]**	**Modulatory Mechanisms**	
GM-CSF	D[3]	*Lactobacillus reuteri, Enterococcus faecalis, Lactobacillus crispatus* and *Clostridium orbiscindens*	P[4]	Reduced expansion and activation of MDSCs by IL-17A-induced release of GM-CSF	Menetrier-Caux et al., 1998; Brown et al., 2017
IL-4	I[5]	*Lactobacillus* spp.	P	Reduced suppressive activity of MDSCs on anti-tumor T-cells by decreasing the expression of IL-4 and IL-13 and subsequent downregulation of arginase 1 expression	Rutschman et al., 2001; Bronte et al., 2003; Johansson et al., 2012
Arginine availability	–	Diverse	P	-Reduced availability of arginine through the depletion of dietary arginine in gut. -Increased radio sensitization of arginine-depleted cancer cells. -More efficient arginine deprivation therapy. -Induced autophagy and apoptosis in arginine auxotrophic cancer cells.	Syed et al., 2013; Hinrichs et al., 2018; Zou et al., 2019
Arginine and endogenous nitric oxide availability	–	*Prevotella* spp.	P	-Reduced availability of arginine and its lower subsequent conversion into nitric oxide. -Improved activity of anti-tumor T-cell by inhibiting MSDCs expansion.	Dai et al., 2015; Kao et al., 2015
Nitric oxide	–	–	P	-Conversion of dietary arginine into nitric oxide. -Increased permeability of mitochondrial membrane and subsequent promoted release of cytochrome c, expression of apoptosis inducing factor, and activation of certain caspases at high level of nitric oxide. -Nitric oxide-induced DNA damage and cell death in cancer cells. -Increased sensitization of resistant tumor cells to apoptosis during chemo-immunotherapy in the presence of nitric oxide.	Sarti et al., 2012; Bonavida and Garban, 2015; Chang et al., 2015; Tengan and Moraes, 2017
			N[6]	-Conversion of dietary arginine into nitric oxide. -Increased MSDCs expansion and subsequent decreased activity of anti-tumor T-cells.	
Peroxynitrite	I	–	N	-Conversion of dietary arginine into nitric oxide and the subsequent formation of peroxynitrite upon reaction with superoxide radicals. -Increased MSDCs-mediated suppressive activity on T-cells. -Promoted tumor progressions by rendering T-cell unresponsive due to aberrant nitration of the T-cell receptor and $CD8^+$ molecules in the presence of excessive amounts of peroxynitrite.	Nagaraj et al., 2007; Gabrilovich and Nagaraj, 2009 Bentz et al., 2000; Cobbs et al., 2003; Gabrilovich and Nagaraj, 2009
Polyamines availability	–	–	N	Conversion of arginine into polyamines which may induce the proliferation and metastasis of tumor cells.	Dai et al., 2017; Zou et al., 2019
Antioxidants	–	Diverse	P	-Reduced suppressive activity of MDSCs on anti-tumor T-cells by some microbial metabolites such as vitamins, antioxidants, and polyphenols that scavenge ROS.	Dehhaghi et al., 2018a, 2019a

(Continued)

TABLE 1 | Continued

Factor	A[1]	Presence of gut microbiota			References
Tryptophan availability	–	*Burkholderia, Ralstonia, Klebsiella, Citrobacter*, and *Bifidobacterium infantis*	N	-Assimilation of dietary tryptophan which causes an aberrant proliferation and functions of effector T-cells due to the inhibition of fatty acid synthesis in human primary CD4+ T-cells by overactivating GGN2 -Induced tumor immunoresistance and survivability by the phosphorylation of eukaryotic initiation factor 2α	Ye et al., 2010; Eleftheriadis et al., 2015; Schalper et al., 2017; Kaur et al., 2019
IDO-1 IFN-γ	I	–	N	-Overactivation of AhR by tryptophan-derived metabolites of gut microbiota (e.g., kynurenine, kynurenic acid) -Ligand-activated AhR eventually increases IDO-1 by increasing the release of IL-6 and IFN-γ.	Glauben et al., 2006; DiNatale et al., 2010; Litzenburger et al., 2014; Wang et al., 2014; Dehhaghi et al., 2018a, 2019a; Martin-Gallausiaux et al., 2018; Kaur et al., 2019
			P	-Inhibited IFN-γ and IDO-1 expression by microbial SCFAs through their downregulatory effects on STAT1 and histone deacetylase	
IDO-1	I	–	N	-Increased pathogenesis of gliomas by overactivating IDO-1 through the production of inflammatory cytokines, amyloid peptide, and lipopolysaccharides. -IDO-1-suppressed expansions of T-cells and other immune cells *via* tryptophan depletion route (see above)	–
IFN-γ	I	–	N	-Weak determinant for impacting T-cell suppressive potency, accumulation, or phenotype of MDSCs – Increases cancer pathogenesis through modification of kynurenine pathway at IDO-1 step (see above).	–
Microglia dysbiosis	I	Diverse for example *Clostridium* spp.	N	-Microbial SCFAs induce the release of TGF-β that triggers dysbiosis of Th1 and Th2 in favor of microglia M2c phenotype and inhibits cytokine production, lymphocyte proliferation, and T cell differentiation.	Mantovani et al., 2004; Heijtz et al., 2011; Erny et al., 2015; Bauché and Marie, 2017; Dehhaghi et al., 2018a, 2019a, 2018b; Roesch et al., 2018; Mehrian-Shai et al., 2019
		–	N	Induced MDSCs suppressive activity on anti-tumor T-cells due to a higher production of ROS in the brain by activated and inflamed microglia in response to microbiota-derived neurotoxic substances or metabolites (e.g., amyloid proteins and LPS)	

[1]*Anti-cancer impact by gut microbiota.*
[2] *Effect on the brain cancer development.*
[3]*Decreased.*
[4]*Increased.*
[5]*Positive.*
[6]*Negative.*

could increase the permeability of the mitochondrial membrane that subsequently triggers the release of cytochrome c, the expression of an apoptosis inducing factor, and the activation of certain caspases (Sarti et al., 2012; Tengan and Moraes, 2017). Interestingly, cancer cells are more susceptible to cytotoxic effects (DNA and mitochondria damage) of nitric oxide due to aberrant P53 protein, compared to normal cells (Chang et al., 2015). Moreover, the sensitization of resistant tumor cells to apoptosis during chemo-immunotherapy, increases in the presence of nitric oxide (Bonavida and Garban, 2015).

It should be noted that arginine starvation can have both positive and negative effects on tumors. Like arginase 1 in MDSCs, the assimilation of L-arginine by gut microbiota will lead to its depletion from the tumor microenvironment. This phenomenon has an inhibitory effect on T-cell proliferation by suppressing T-cell cell cycle regulators (cyclin-dependent kinase 4 and cyclin D3) and downregulating the expression of T-cell antigen, i.e., CD3 zeta(ζ)-chain (Rodriguez et al., 2002, 2007). Accordingly, the failure of T-cells to upregulate the mentioned cell regulators, arrests T-cell in the G_0-G_1 phase of the cell cycle. This is done through aberrant downstream signaling, i.e., a blocked GCN2 signaling pathway that involves a lower level of Rb protein phosphorylation, and weak E2F1 expression and binding.

On the positive side, the depletion of nutritional arginine by gut microbiota can be beneficial for eradication of arginine auxotrophic tumors, which lack the enzyme (i.e., argininosuccinate synthetase) required for the conversion of citrulline to arginine (Feun et al., 2008; Syed et al., 2013; Khoury et al., 2015; Zou et al., 2019). Therefore, these types of tumor cells must rely on the exterior source of arginine to meet their extremely high metabolic rate and intensive growth. Even the sensitization of nonauxotrophic glioblastoma is induced in the absence of arginine (Hinrichs et al., 2018). Under arginine depletion stress, tumor cells switch to autophagy to maintain their functions, but eventually undergo apoptosis due to excessive autophagy.

Tryptophan

Tryptophan is an essential amino acid, which means it cannot be synthesized endogenously in the human body and must be supplied through dietary intake. Two forms of tryptophan, i.e., either bound to albumin or free form, can be found in the human body (Dehhaghi et al., 2019a). Only the free form of tryptophan can across the BBB through non-specific L-amino acid transporters. Although the level of tryptophan in human tissues is lower than other amino acids, it is a critical component of several metabolic pathways. After tryptophan uptake, it participates in protein synthesis or enters various metabolic pathways based on the tissue and the enzymes expressed. While approximately, 90% of tryptophan is metabolized through the kynurenine pathway, generating various neuroactive metabolites in the body, 3–10% of tryptophan is utilized for the synthesis of chemical messengers such as serotonin, tryptamin, and other indole-derived metabolites (Palego et al., 2016; Dehhaghi et al., 2019a). Biotransformation of tryptophan and its availability is regulated by both endogenous and exogenous factors. The association of tryptophan depletion with some neurological disorders such as depression and mood-affective diseases are well-known (Kanchanatawan et al., 2018).

With respect to cancers, tryptophan depletion is increasingly being identified as a critical factor in tumor cell development. Importantly, tryptophan is highly catabolized in a microenvironment of tumor cells and inflammation regions. This local tryptophan depletion promotes T-cells to activate general control nonderepressible-2 (GCN2) kinase, i.e., a serine-threonine kinase that responds to amino acid deprivation. Consequently, a stress response is induced by activated GCN2 that leads to an aberrant proliferation of effector T-cells (Schalper et al., 2017). More specifically, activated GCN2 is able to inhibit fatty acid synthesis in human primary $CD4^+$ T-cells that is essential for their regular proliferations and functions (Eleftheriadis et al., 2015). It is worth mentioning that $CD4^+$ T-cells show a reduced survivability in the presence of kynurenine under tryptophan-depleted locations. In tumor cells, abnormal angiogenesis is associated with the lack of blood supply, which is directly related to the development of hypoxia and the deprivation of glucose, amino acids, and other essential nutrients. Under this condition, the phosphorylation of eukaryotic initiation factor 2α (eIF2 α) occurs by activation of GCN2, which upregulates activating transcription factor 4 (ATF4) expression, increases amino acid biosynthesis, and eventually induces tumor immunoresistance and its survival (Ye et al., 2010). Additionally, IDO-1 expressing tumor cells respond to tryptophan shortage by expressing amino acid transporter genes (i.e., *SLC7A11, SLC1A4*, and *SLC1A5*). The upregulation of ATF4-dependent expression of *SLC1A5* and its splice variants eventually increase glutamine and tryptophan uptake that are highly demanded for rapid amino acids synthesis in tumor cells (Timosenko et al., 2016).

Expression of IDO-1 in non-antigen-presenting cells, such as tumor cells, promotes the escape of the tumors from immunosurveillance (Munn and Mellor, 2007). Generally, IDO-1 can inhibit glucose uptake, glycolysis, and glutaminolysis, which contribute in its immunosuppressive activity (Eleftheriadis et al., 2013). Like activated T cells, highly proliferating cells such as cancer cells change their metabolic pathways from pyruvate oxidation to the glycolytic and glutaminolytic pathways. Cancerous cells showed Warburg's phenomenon in which is an enhanced cytoplasmic glycolysis/mitochondrial oxidation ratio (Warburg, 1956; Wang et al., 2011). Most cancer cells can directly or indirectly affect the function of p53 (a potent tumor suppressor) that inhibits cell proliferation by arresting the cell cycle in G1-phase. In addition, p53 plays significant role in the modulation of cellular metabolism through inhibiting glucose uptake and aerobic glycolysis (Brady and Attardi, 2010; Shen et al., 2012). With respect to the similarity in glucose metabolism in activated T cells and cancer cells, it has been shown that IDO-induced tryptophan depletion increased p53 level. Both IDO-1 and p53 inhibited the aerobic glycolysis in alloreactive T cells (Eleftheriadis et al., 2014). IDO-induced tryptophan depletion also activated the GCN2 kinase leading to p53 up-regulation in T cells (Eleftheriadis et al., 2014).

Based on *in silico* analyses, bacterial phyla residing in the human GI tract including Actinobacteria, Bacteroides,

Firmicutes, Fusobacteria, and Proteobacteria possess complex pathways to metabolize tryptophan and produce neuroactive metabolites such as kynurenine, kynurenic acid, quinolinate, tryptamine, indole, and indole-derivatives (DiNatale et al., 2010; Kaur et al., 2019). Some gut bacterial genera (e.g., *Burkholderia*, *Ralstonia*, *Klebsiella*, and *Citrobacter*) have higher potential to convert tryptophan to neuroactive compounds, compared to other bacteria (Kaur et al., 2019). Interestingly, microbial tryptophan-derived indole and indole derivatives could profoundly modulate gut homeostasis and cellular gene expression. Moreover, indolyl metabolites may be significant signaling molecules between the gut and immune system as they can bind to the aryl hydrocarbon receptor (AhR) and activate it locally and systemically (Cheong and Sun, 2018; Dehhaghi et al., 2019a). More importantly, gut-microbiota can affect the disease state of cancer by modulating IDO-1 activity (see Section "Kynurenine Pathway") through regulating AhR activity. The interaction of some tryptophan-derived gut microbiota with AhR causes its activation. Ligand-activated AhR then regulates the functions of a wide range of innate and adaptive immune system such as dendritic cells, natural killer cells, macrophages, regulatory T-cells, and type 17 and 22 helper T-cells (Cheong and Sun, 2018). The dissociation of AhR from the chaperone heat shock protein 90 occurs after binding ligands to AhR. The ligand-activated AhR translocates nucleus and forms a heterodimeric complex with the AhR nuclear translocator (ARNT) protein. The AhR-ARNT complex is a transcription factor that regulates the expression of IL-6 in macrophages, IL-10 in natural killer cells and dendritic cells (Litzenburger et al., 2014; Wang et al., 2014). IL-6 then activates IDO-1 and indirectly contributes in increased kynurenine and kynurenic acid production and AhR activation. Moreover, AhR activation in natural killer cells induces INF-γ production that subsequently induces IDO-1 expression and eventually leads to tryptophan depletion (**Figure 1**).

Overall, the depletion of tryptophan has pro-cancer activities (**Table 1**) and increases the survivability and severity of tumors. In a tryptophan-depleted microenvironment, the responses of antigen-specific T-cells are suppressed through accumulation of tryptophan-derived immunosuppressive metabolites (Mellor and Munn, 2008). As tryptophan is essentially provided through the diet, gut microbiota may help cancer cells to evade immunity in the human body through assimilation of available tryptophan in the gut.

KYNURENINE PATHWAY

Tryptophan, an essential amino acid for animals and humans, is mainly supplied through dietary nutrient intake. The kynurenine pathway is the main route of tryptophan catabolism, contributing to *de novo* synthesis of nicotinamide adenosine dinucleotide (NAD) through the production and conversion of various neuroactive intermediates (**Figure 2**; Chen and Guillemin, 2009; Dehhaghi et al., 2019a). The heme-enzymes IDO-1, indoleamine 2,3-dioxygenase-2 (IDO-2), and tryptophan 2,3-dioxygenase (TDO-2) are three regulatory enzymes of the kynurenine pathway that catalyze the first step of tryptophan degradation. TDO and IDO are predominantly expressed in liver and various cells (e.g., intestinal cells, microglia, astrocytes, macrophages, and neuronal cells), respectively (Ball et al., 2014). IDO-1 expression is significantly induced by interferon gamma (INF-γ). However, other inflammatory cytokines, amyloid peptide, lipopolysaccharides, and TLR ligands could also induce its expression (Guillemin et al., 2003; Adams et al., 2012). The kynurenine pathway-derived metabolites, particularly kynurenic acid and quinolinic acid, could potentially have neuroprotective and neurotoxicity effects on the neuronal activity in the central and peripheral nervous systems, respectively (Carpanese et al., 2014; Filpa et al., 2015). Therefore, an aberrant kynurenine pathway metabolism could be triggered by unbalanced gut microbiota.

Clinical studies have suggested IDO-1 activity could be associated with various cancers including melanoma, colorectal, breast, lung, and brain cancers (Ferdinande et al., 2012; Zhai et al., 2015, 2018). Glioma and glioneuronal tumors show increased tryptophan uptake and catabolism. Compared to control healthy cells, the activity of IDO-1 is induced in malignant glioma cells due to the presence of INF-γ (Adams et al., 2012). This could induce pro-cancer activities through depletion of tryptophan (see Section "Tryptophan"). The upregulation of IDO-1 mRNA levels is positively correlated with the glioma grade, while it has an inverse relationship with the survival rate in patients with gliomas (Zhai et al., 2017). In fact, the overexpression of IDO-1 in cancer cells helps to evade immune surveillance by suppressing the expansion of T-cells and other immune cells.

Gut microbiota can influence the kynurenine pathway through the regulation of immune system-associated IDO-1 activity or the modulation of tryptophan availability. For instance, the levels of tryptophan circulation increase in the absence of gut microbiota (in germ-free models) or altered microbial composition induced by antibiotics (Dehhaghi et al., 2019a). This aberrant tryptophan level further decreases kynurenine-to-tryptophan ratio in plasma by modifying IDO-1 activity. Unlike the circulatory tryptophan level, both kynurenine metabolites and the peripheral serotonin level decrease in germ free animals (Clarke et al., 2013; Yano et al., 2015). It is important to note that the introduction of some gut microorganisms, for example *Bifidobacterium infantis*, can restore the normal kynurenine-to-tryptophan ratio (Desbonnet et al., 2008). This is because gut microbiota is capable of degrading tryptophan to various metabolites that consequently limit the availability of tryptophan for the kynurenine pathway and other tryptophan catabolism routes such as the serotonin production pathway.

Interestingly, IDO-1 and gut microbiota have feedback control on each other. While IDO-1 can induce an immunosuppressive response in the GI tract through the regulation of microbial metabolism and immune reactivity, gut microbiota can alter the kynurenine pathway and IDO-1 activity by affecting tryptophan availability (Dehhaghi et al., 2019a). Moreover, molecular analyses of bacterial genomes have revealed homologs of TDO, 3-hydroxyanthranilate-3,4-dioxygenase, kynurenine-3 monooxygenase, and kynureninase. These genes confer gut microbiota the potential to produce neuroactive metabolites.

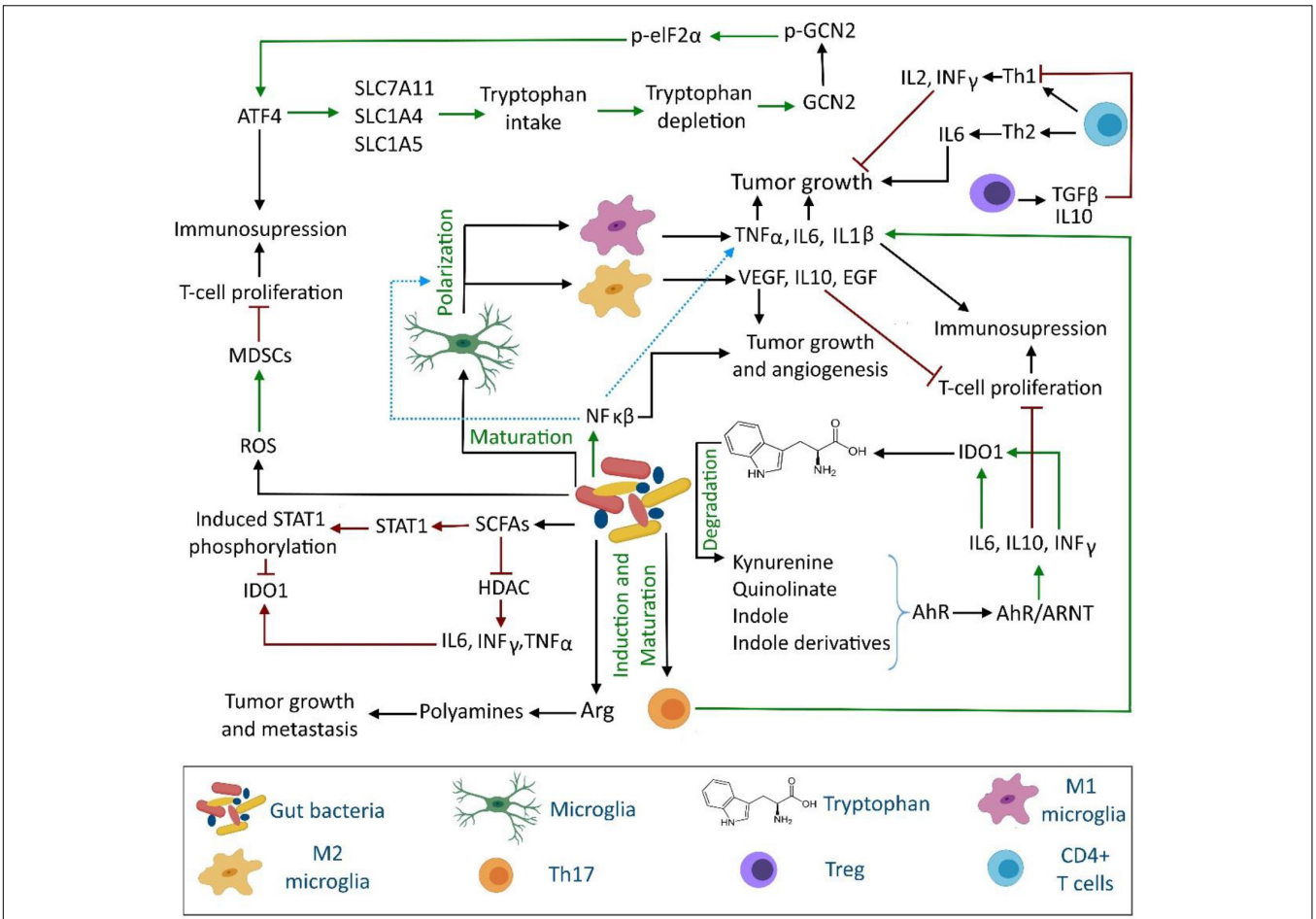

FIGURE 1 | Interaction between gut microbiota, immune system and tryptophan metabolism in brain tumor development. The gut microbiota can influence the brain tumor cells proliferation and metastasis through direct and indirect regulation of tryptophan and arginine metabolism, inflammatory cytokine production, release of short chain fatty acids, aryl hydrocarbon receptor modulation, microglia maturation, and T cell proliferation. AhR, aryl hydrocarbon receptor; Arg, arginine; ARNT, AhR nuclear translocator; ATF4, activating transcription factor 4; eIF2α, eukaryotic initiation factor 2α (eIF2 α); GCN2, general control nonderepressible-2; HDAC, histone deacetylase; MDSCs, myeloid-derived suppressor cells; ROS, reactive oxygen species; SCFAs, short chain fatty acids; STAT, signal transducer and activator of transcription.

The microbial products of these homologs can influence the expression and activity of IDO-1.

Some microbial derived metabolites in GI such as SCFAs exert an anti-inflammatory effect and modulate the immune system and kynurenine pathway through regulation of IDO-1 activity. Generally, the interaction between cells and SCFAs activates a signaling cascade that involve G-protein coupled receptors (GPR41, GPR43, and GPR109a) (Brown et al., 2003; Dehhaghi et al., 2018a). SCFAs (especially, butyrate) that are produced by gut microbiota play a significant role in intestinal homeostasis and cancer protection. It has been demonstrated that butyrate-associated IDO-1 regulation is mainly conducted by two mechanisms that are independent of the known G-protein coupled receptors (i.e., GPR41, GPR43, and GPR109a) for SCFAs (Martin-Gallausiaux et al., 2018). The first mechanism involves the downregulation of the expression of signal transducer and activator of transcription (STAT) 1, which is a main mediator of IDO-1 expression. Decreased STAT1 expression inhibits INF-γ-dependent STAT1 phosphorylation, and subsequently reduces STAT1-dependant transcriptional activity of IDO-1. In the second mechanism, IDO-1 activity is affected through a STAT1-independent route for the inhibition of IDO-1 activity. In this mechanism, IDO-1 activity is regulated by SCFAs through their histone deacetylase (HDAC) inhibitory properties (Dehhaghi et al., 2018a). The inhibition of HDAC could suppress the production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IFN-γ, and IL-6 and could subsequently inhibit IDO-1 activity (Glauben et al., 2006).

GUT DYSBIOSIS AND MICROGLIAL DYSFUNCTION

Immunity responses in the brain are controlled by microglial cells, i.e., resting microglia and activated microglia (viz., proinflammatory microglia, M1 phenotype; and

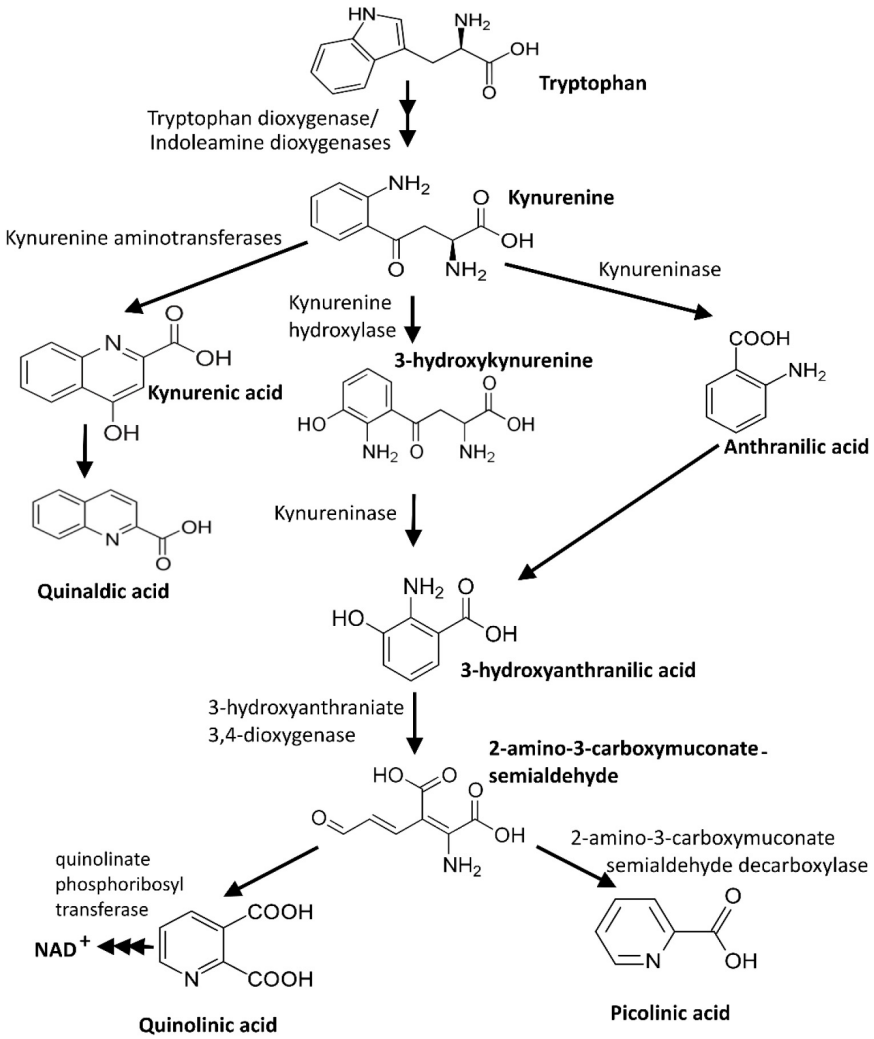

FIGURE 2 | Kynurenine pathway, the main route of tryptophan catabolism (Dehhaghi et al., 2019a).

immunosuppressive and tissue-regenerating phenotype microglia, M2 phenotype). The polarization process could be controlled by stimulus, period, and environment, which modulate the expression of CD86, CD45, MHC class II in microglia (Ma et al., 2017). More specifically, the resting microglia could be activated and polarized toward M1 phenotype through stimulation by trauma-induced cellular debris, bacterial-derived compounds (e.g., lipopolysaccharide), cytokines produced by type 1 T helper (Th1) cells and astrocytes (e.g., interferon-γ, and TNF-α). Many of these compounds are also produced or induced by gut microbiota (Heijtz et al., 2011; Dehhaghi et al., 2018a). M1 phenotype produces (i) proteolytic enzyme matrix metalloproteinase-3 and matrix metalloproteinase-9, (ii) redox signaling molecules such as reactive oxygen species and nitric oxide, and (iii) several proinflammatory cytokines, including IL-1ß, IL-6, targeting chemokine (C-C motif) ligand 2, TNF-α, C-X-C motif chemokine 10. Normally over time, type 2 T helper (Th2) cells stimulate the polarization of microglia into M2 phenotype through the production of IL-4 and IL-13. Unlike M1, M2 microglia produce scavenger receptors and anti-inflammatory cytokines, including tumor necrosis factor-β (TGF-β), insulin-like growth factor 1, IL-4, IL-10, and IL-13. Three phenotypes of M2 activated microglia function in inflammation suppression (i.e., M2a sub-class) and tissue regeneration (M2c sub-class), while M2b still has an unknown role (Latta et al., 2015; Mecha et al., 2015).

Therefore, Th 1 and Th 2 cells could mediate microglial phagocytosis, and their production of cytokines and other effector molecules that ultimately suppress the cause of inflammation. The disruption of these control mechanisms; however, could cause autoimmune diseases as well as brain cancer. The latter complication may be attributed to the unregulated tissue reconstruction triggered by activated M2 microglia. Switching to anti-inflammatory M2 phenotype, specifically M2c subtype, is induced by cytokines and

corticosteroids produced by tumor cells, such as IL-10, TGF-β, and glucocorticoids. Accordingly, M2c phenotype is a deactivated form of microglia which is associated with tumor growth and brain malignancies (Mantovani et al., 2004; Roesch et al., 2018). More specifically, human high-grade gliomas have overexpressed amounts of cytokine colony stimulating factor 1 (CSF1), CSF1 receptor, and cytokines (i.e., IL-6 and TGF-β). It is worth mentioning that CSF1 is produced by microglia and macrophages, whereas IL-6 and TGF-β are released by Th 2 cells. TGF-β strongly inhibits cytokine production, lymphocyte proliferation, and T cell differentiation. In contrast, IL-2 and IL-12 are released from Th 1 cells in normal tissues and are absent in glioblastoma tumors (Mehrian-Shai et al., 2019; **Figure 1**).

Further, intestine epithelial cells produce high levels of TGF-β that is controlled by gut microbiota. For example, microbial fermentation products such as SCFAs (i.e., butyrate, acetate, and propionate) could be produced by *Clostridium* spp. (Dehhaghi et al., 2019a) even in gut, promoting TGF-β production (Bauché and Marie, 2017; Dehhaghi et al., 2019a). While the role of TGF-β in neuronal and glial cell development has been well documented, gut microbiota may play a crucial role in microglial maturation and function through controlling TGF-β production. This hypothesis has been supported by an *in vivo* study involving germ-free mice (Erny et al., 2015). Compared to the control normal mice, germ-free mice showed substantial changes in the properties of the microglia, including alteration in morphological features, gene expression, and the maturation process. Additionally, microbiota diversity is also correlated with the maturation of microglia. In fact, limited gut microbiota diversity could result in defective microglia (i.e., the number of immature microglia in the brain cortex increased) (Erny et al., 2015). It is worth mentioning that gut microbiota may also increase the severity of cancer by the production of neurotoxic substances (e.g., amyloid proteins, and lipopolysaccharides) that could pass BBB. Once in the brain, these microbial compounds increase ROS production by the activation and inflammation of microglia (Dehhaghi et al., 2018a). The induced inflammation then may trigger IDO-1 overactivation (see Section "Kynurenine Pathway"), tryptophan depletion (see Section "Tryptophan"), or MDSCs activation (see Section "Myeloid-Derived Suppressor Cells").

MYELOID-DERIVED SUPPRESSOR CELLS

MDSCs are a heterogenous population of immunosuppressive cells that originate from immature myeloid cells. Following immature myeloid cells generation in bone marrow, they are released for their subsequent differentiation into mature myeloid cell, i.e., dendritic cells, macrophages, or granulocytes in peripheral organs. Unlike in a steady state, a partial inhibition in the differentiation of immature myeloid cells into mature myeloid cells occurs during various pathological conditions including cancer, and to a significantly lesser extent, infection, inflammation, sepsis, and trauma (Gabrilovich and Nagaraj, 2009; Raychaudhuri et al., 2015; Alban et al., 2019). These diseases expand MDSC in the circulatory system of patients by modifying STAT3 and Janus protein family members (Bromberg, 2002). Moreover, during pathological conditions, the activated immature myeloid cells remarkably express reactive oxygen species (ROS), nitrogen species (i.e., inducible nitric oxide synthase (iNOS) and nitric oxide), arginase 1, and other immune suppressive factors that increase the suppressive activity of MDSCs on T-cells in lymphoid organs, circulation, and tumors (in case of cancer) (Gabrilovich and Nagaraj, 2009).

In humans, $CD11b^{+}CD14^{-}CD33$, $HLA\text{-}DR^{-}$-CD33, or $HLA\text{-}DR^{-}$-$CD15^{+}$ phenotypes of MDSCs have been identified (Gabrilovich and Nagaraj, 2009). MDSCs have the morphology of monocytes or granulocytes and can significantly suppress T-cell responses. Up to 5.4% of the total cells in GBM tumors is made up of MDSCs that mainly include lineage negative ($CD14^{-}CD15^{-}$), followed by granulocytic ($CD14^{-}CD15^{+}$) and monocytic ($CD14^{+}CD15^{-}$) subtypes (Raychaudhuri et al., 2015). The accumulation of MDSCs in GBM tumors attracts T regulatory cells but suppresses anti-tumor T-cell proliferation and functions. A positive correlation exists between the intratumoral density of glioma-associated MDSCs, and the patient's survival and histological grade of gliomas (Gieryng and Kaminska, 2016). Intriguingly, T cell effector responses that are required for tumor rejection could be restored when MDSCs are removed (Movahedi et al., 2008; Gabrilovich and Nagaraj, 2009).

One of the promising methods for reducing the population of MDSCs is through enriching gut microbiota that increases granulocyte–macrophage colony-stimulating factor signaling (GM-CSF) (Brown et al., 2017). Moreover, gut microbiota can block STAT6 in MDSC by decreasing the expression of IL-4 that is important for signaling downstream of IL-4 receptor α-chain. In fact, this deficient signaling pathway prevents both the activity and production of arginase 1, which in turn, decrease the T-cell suppressive function of MDSCs (Rutschman et al., 2001; Gabrilovich and Nagaraj, 2009). It is worth mentioning that the IL-4 receptor α-chain-STAT6 pathway does not induce tumor immunosuppression in all tumor microenvironment.

Gut-microbiota can also decrease the suppressive activity of MDSCs through modification of ROS level. The generation of the ROS may occur during physiological processes (i.e., endogenous ROS), interaction with external harmful stress (exogenous ROS) such as ionizing radiation, or in a response to disease state (Dehhaghi et al., 2019c). ROS examples include nitric oxide and peroxynitrite, singlet oxygen, hydroxyl radicals, superoxide, and peroxides (Dehhaghi et al., 2019c). The speculative intervention of gut microbiota in the ROS level of the brain could be through the production of microbial metabolites (Bonaz et al., 2018; Dehhaghi et al., 2019a). Some of these metabolites such as SCFAs (see Section "Kynurenine Pathway"), vitamins, antioxidants, and polyphenols inhibit the ROS. The existence of gut microbiota could suppress extensive colonization of pathogens in the gastrointestinal tract while could also regulate the immune response and the permeability of BBB and intestinal barrier (Dehhaghi et al., 2018a). Some of these modifications and interferences may lower the generation of ROS in the body, even in the brain, and hence, inhibit MSDCs suppressive activity. Gut microbiota could also manipulate central nervous

system ROS flux through providing extra amounts of some neurotransmitters (e.g., gamma-amino butyric acid, serotonin, and dopamine) and/or through kynurenine pathways (see Section "Tryptophan").

On the downside, gut microbiota may also increase the severity of cancer through activation of microglia and the subsequent induction of ROS generation (see Section "Myeloid-Derived Suppressor Cells"). Gut microbiota could also increase the flux of nitric oxide (see Section "Arginine") and peroxynitrite in the body and the brain. Excessive amounts of peroxynitrite render T-cells unresponsive, which could be associated with the tumor progressions in many cancer types (Bentz et al., 2000; Cobbs et al., 2003; Gabrilovich and Nagaraj, 2009). This could be attributed to the ability of peroxynitrite for modifying the antigen-specific stimulation response of T-cells due to aberrant nitration of the T-cell receptor and $CD8^+$ molecules (Nagaraj et al., 2007; Gabrilovich and Nagaraj, 2009).

Alternatively, gut microbiota can induce the expression and activation of INF-γ following the assimilation of gluten which cannot be completely digested by humans (Dehhaghi et al., 2019a). INF-γ may inhibits anti-tumor T-cells through the expression of iNOS, arginase by MDSCs in the tumor microenvironment. However, further study has shown that although INF-γ has the potential to signal through the STAT1 pathway, it is not a key determinant for impacting T-cell suppressive potency, accumulation, or phenotype of MDSCs (Sinha et al., 2012). Therefore, gut microbiota-induced IFN-γ probably increases cancer pathogenesis through modification of the kynurenine pathway at IDO1 step (see Section "Kynurenine Pathway").

CONCLUSION

Gut-brain axis may negatively or positively influence cancer development, including brain cancer, through the production of various metabolites. These metabolites may induce or suppress the release of specific cytokines, which could trigger inflammatory or anti-inflammatory responses. The intervention of gut microbiota in cancer development can be both negative and positive. For example, gut microbiota can modulate suppressive activity of MDSCs on anti-tumor T-cells by producing pro-inflammatory (e.g., amyloid proteins and lipopolysaccharides) and anti-inflammatory substances (e.g., antioxidants, polyphenols, and vitamins). With respect to anti-tumor activities, some gut microorganisms such as *Lactobacillus reuteri, Enterococcus faecalis, Lactobacillus crispatus* and *Clostridium orbiscindens* can induce the differentiation of MDSCs into mature myeloid cells. This is done by mediating the release of GM-CSF that is controlled by IL-17A. Individuals with a high population of *Lactobacillus* spp. in their GI track show a lower expression of IL-4 that suppressed the activity of MDSCs on anti-tumor T-cells by downregulating arginase 1 expression.

Through depletion of some dietary amino acids, gut microbiota can modulate the tumor microenvironment. On this basis, the assimilation of dietary arginine in the gut by microorganisms (e.g., *Prevotella* spp.) reduces flux in the human body that favors anti-tumor activities. Under an arginine depleted condition, arginine-auxotrophic cancers cells must extensively rely on autophagy to meet their rapid metabolisms that eventually trigger apoptosis. As a result of reduced arginine flux, a lower level of nitric oxide is synthesized by the body that inhibits the expansion of MSDCs, and hence, improves the activity of the anti-tumor T-cell. However, microbial derived nitric oxide could be also produced during the assimilation of dietary arginine by gut microbiota. The resultant nitric oxide could then form peroxynitrite upon its reaction with superoxide radicals. Both nitric oxide and peroxynitrite can induce tumor development by increasing MSDC-mediated suppressive activity on T-cells, whereas polyamines (another group of arginine-derived microbial compounds in the gut) induce the proliferation and metastasis of tumor cells. Alternatively, peroxynitrite causes pro-cancer activity through rendering T-cells unresponsive due to aberrant nitration of the T-cell receptor and CD8+ molecules. Nevertheless, it should also be noted that tumor cells are more prone to nitric-oxide-mediated cellular damages (i.e., mitochondria and DNA) and apoptosis due to their aberrant P53 pathway.

Unlike arginine, the depletion of tryptophan by gut microbiota (e.g., *Burkholderia, Ralstonia, Klebsiella, Citrobacter*, and *Bifidobacterium infantis*) induced tumor immune-resistance and survivability by the phosphorylation of eukaryotic initiation factor 2α. Moreover, fatty acid synthesis in human primary CD4+ T-cells is disrupted due to the overactivation of GGN2 in tryptophan depleted environments. This leads to aberrant proliferation and functions of effector T-cells in favor of tumor cells. Gut microbiota may also convert tryptophan into kynurenine and kynurenic acid that over-activate AhR. Ligand-activated AhR then can induce IDO-1 activity through promoting the release of IL-6 and INF-γ. IDO-1 could also be overactivated due to the production of inflammatory cytokines, amyloid peptide, and lipopolysaccharides. Interestingly, IDO-1 overactivation leads to further tryptophan depletion. In contrast, gut microbiota may also produce anti-inflammatory products such as SCFAs that inhibit both INF-γ and IDO-1 by downregulating STAT1 and histone deacetylase. However, SCFAs could also induce the release of TGF-β that triggers dysbiosis of Th1 and Th2 in favor of microglia M2c phenotype and inhibits cytokine production, lymphocyte proliferation, and T cell differentiation.

AUTHOR CONTRIBUTIONS

MD and HK wrote the manuscript and prepared the figures. GG and BH have reviewed and corrected the manuscript based on their respective expertise. All authors read and approved the final version of the manuscript.

FUNDING

MD and HK were both supported by an International Ph.D. Scholarship from Macquarie University. BH was supported by

Tour de Cure and Fight on the Beach Foundation. GG was supported by the National Health and Medical Research Council (NHMRC), the Australian Research Council (ARC), the Handbury Foundation, and Macquarie University.

ACKNOWLEDGMENTS

This manuscript was professionally edited by Red Fern Communication, Sydney, NSW, Australia.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 11 December 2020
doi: 10.3389/fcimb.2020.585973

A Metagenome-Wide Association Study of Gut Microbiome in Patients With Multiple Sclerosis Revealed Novel Disease Pathology

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While microbiome plays key roles in the etiology of multiple sclerosis (MS), its mechanism remains elusive. Here, we conducted a comprehensive metagenome-wide association study (MWAS) of the relapsing-remitting MS gut microbiome (n_{case} = 26, $n_{control}$ = 77) in the Japanese population, by using whole-genome shotgun sequencing. Our MWAS consisted of three major bioinformatic analytic pipelines (phylogenetic analysis, functional gene analysis, and pathway analysis). Phylogenetic case-control association tests showed discrepancies of eight clades, most of which were related to the immune system (false discovery rate [FDR] < 0.10; e.g., *Erysipelatoclostridium_sp.* and *Gemella morbillorum*). Gene association tests found an increased abundance of one putative dehydrogenase gene (Clo1100_2356) and one ABC transporter related gene (Mahau_1952) in the MS metagenome compared with controls (FDR < 0.1). Molecular pathway analysis of the microbiome gene case-control comparisons identified enrichment of multiple Gene Ontology terms, with the most significant enrichment on cell outer membrane ($P = 1.5 \times 10^{-7}$). Interaction between the metagenome and host genome was identified by comparing biological pathway enrichment between the MS MWAS and the MS genome-wide association study (GWAS) results (i.e., MWAS-GWAS interaction). No apparent discrepancies in alpha or beta diversities of metagenome were found between

OPEN ACCESS

Edited by:
Paola Brun, University of Padua, Italy

Reviewed by:
Ashu Sharma, University at Buffalo, United States
Ashutosh K. Mangalam, The University of Iowa, United States

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Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *22 July 2020*
Accepted: *13 November 2020*
Published: *11 December 2020*

Citation:
Kishikawa T, Ogawa K, Motooka D, Hosokawa A, Kinoshita M, Suzuki K, Yamamoto K, Masuda T, Matsumoto Y, Nii T, Maeda Y, Nakamura S, Inohara H, Mochizuki H, Okuno T and Okada Y (2020) A Metagenome-Wide Association Study of Gut Microbiome in Patients With Multiple Sclerosis Revealed Novel Disease Pathology. Front. Cell. Infect. Microbiol. 10:585973. doi: 10.3389/fcimb.2020.585973

MS cases and controls. Our shotgun sequencing-based MWAS highlights novel characteristics of the MS gut microbiome and its interaction with host genome, which contributes to our understanding of the microbiome's role in MS pathophysiology.

Keywords: metagenome shotgun sequencing, genome-wide association study, dysbiosis, gut microbiome, multiple sclerosis

INTRODUCTION

The human microbiome is fingerprint of the pathogenesis and therapeutic effect of human complex diseases such as metabolic diseases, immune diseases, and cancer, as well as inflammatory bowel diseases (Forslund et al., 2015; Franzosa et al., 2019; Yachida et al., 2019; Kishikawa et al., 2020a). As illustrated in the word "brain-gut axis," central nervous diseases are closely related to gut microbiome, and the disease mechanism in this context has been elucidated (Cryan et al., 2019). For example, Benakis *et al.* reported that effector T cells transferred from the gut to the brain after stroke enhanced ischemic neuroinflammation by secreting IL-17 (Benakis et al., 2016).

Multiple sclerosis (MS) is the most prevalent chronic immune-related inflammatory disease of the central nervous system, which devastates global health with socioeconomic burdens (Okuno et al., 2015; Reich et al., 2018). The prevalence rate is 50–300 per 100,000 people, with an estimated 2–3 million people globally affected with multiple sclerosis (Thompson et al., 2018). Genome-wide association studies (GWAS) and further fine mapping have discovered many MS-associated genomic regions so far and a part of its pathogenesis has been elucidated (International Multiple Sclerosis Genetics Consortium, 2019; Ogawa et al., 2019). Of note, the number of patients affected with MS in Japan has increased approximately four times in the last 30 years (Osoegawa et al., 2009), which indicates that there exists an emerging risk of developing MS other than the genetic factor. Gut microbiome is considered as one of the candidate risk factors of MS. So far, multiple MS-associated bacteria have been reported, and the biological effects of those bacteria on MS etiology have been demonstrated by using disease models such as experimental autoimmune encephalomyelitis (EAE) (Jangi et al., 2016; Berer et al., 2017; Cekanaviciute et al., 2017).

There exist two major analytical approaches utilizing next generation sequencing technologies in the field of microbiome studies; the classical one is 16S ribosomal RNA (rRNA) sequencing dealing with a part of the microbiome, and the advanced one is whole-genome shotgun sequencing (Ranjan et al., 2016). 16S rRNA sequencing can detect only the relative abundance of each taxon, not the biological functions. The taxonomic assessment is limited at the genus level, and less precise at the species level. On the other hand, whole-genome shotgun sequencing can detect the genomic composition of the microbiome with high resolution at the species level (not only bacteria but also archaea, fungi, and viruses) without bias induced from PCR and individual differences in the number of 16S rRNA. In addition, this novel method has another benefit of analyzing microbiome's biological functional features, by conducting metagenome-wide association study (MWAS; (Kishikawa et al., 2020a; Kishikawa et al., 2020b; Zhu et al., 2020). While MWAS is a powerful to disentangle disease-related pathophysiology of microbiome, requirements of relatively high costs, computational resources to analyze large datasets of next generation sequencing, and complicated data analysis protocols have hampered its application. There exist few MWAS of MS based on whole-genome shotgun sequencing yet (Ventura et al., 2019).

Here, we report a comprehensive MWAS of the gut microbiome in a relapsing-remitting MS (RRMS) case-control cohort of the Japanese population. We carried out whole-genome shotgun sequencing of 103 fecal samples (26 individuals with MS and 77 healthy controls). Our MWAS consisted of three major bioinformatic analytic techniques (phylogenetic analysis, functional gene analysis, and pathway analysis), which allowed us to comprehensively grasp case-control disparity in the gut metagenome. We also compared the pathway enrichment of the gut microbiome MWAS and that of the host GWAS in MS to evaluate the link between the gut metagenome and the human germline genome (i.e., MWAS-GWAS interaction).

METHODS

Patient Participation

We enrolled 28 RRMS patients at Osaka University Hospital. MS patients were diagnosed according to the McDonald 2010 criteria (Polman et al., 2011). The 77 healthy controls were enrolled at Osaka University Graduate School of Medicine. Healthy controls had no personal history of immune-related diseases and treatment with antibiotics for at least one month prior to sampling. Exclusion criteria for both groups were as follows: (i) under 20 years old, (ii) extreme diets (e.g., strict vegetarians), or (iii) treatment with antibiotics for at least 1 month prior to sampling. All the subjects provided written informed consent before participation. The study protocol was approved by the ethical committees of Osaka University and related medical institutions.

Abbreviations: EAE, experimental autoimmune encephalomyelitis; EDSS, Expanded Disability Status Scale; FDR, false discovery rate; GO, Gene Ontology; GSEA, gene set enrichment analysis; GWAS, genome-wide association study; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharides; MWAS, metagenome-wide association study; NGS, next generation sequencing; NMO, neuromyelitis optica; ORFs, open reading frames; PCA, principal component analysis; PERMANOVA, permutational multivariate analysis of variance; QC, Quality control; RRMS, relapsing-remitting multiple sclerosis; rRNA, ribosomal RNA; SCFA, short-chain fatty acid.

Sample Collection and DNA Extraction

For patients, fecal samples had been immediately frozen after production in an insulated container for storage at −20°C and subsequently stored at −80°C within 24 h after production. For healthy controls, samples were stored at −80°C within 6 h after production. Bacterial DNA was extracted according to a previously described method (Maeda et al., 2016; Okumura et al., 2016). Briefly, RNAlater (Ambion) was added to make 10-fold dilutions of homogenates. Three hundred µl of sodium dodecyl sulfate–Tris solution, 0.3 g glass beads (diameter 0.1 mm) (BioSpec), and 500 µl EDTA-Tris-saturated phenol were added to the suspension, and the mixture was vortexed vigorously using a FastPrep-24 (MP Biomedicals) at 5.0 power level for 30 s. After centrifugation at 20,000 g for 5 min at 4°C, 400 µl of supernatant was collected. Subsequently, phenol-chloroform extraction was performed, and 250 µl of supernatant was subjected to isopropanol precipitation. Finally, DNAs were suspended in 200 µl EDTA-Tris buffer and stored at −80°C.

Whole-Genome Shotgun Sequencing

A shotgun sequencing library was constructed using the KAPA Hyper Prep Kit (KAPA Biosystems) and 150 bp paired-end reads were generated on a HiSeq 3000 (average 7.3 Gb per sample). The sequence reads were converted to FASTQ format using bcl2fastq (version 2.19).

Quality Control (QC) of Sequencing Reads and Samples

We applied a series of QC steps to maximize the quality of the datasets. The main QC steps were: (i) trimming of low-quality bases, (ii) identification and masking of human reads, and (iii) removal of duplicated reads. We trimmed the raw reads to clip Illumina adapters, cut off low-quality bases at both ends, and discarded reads less than 60 bp in length after trimming using the Trimmomatic (version 0.33, parameters: ILLUMINACLIP : TruSeq3-PE-2.fa:2:30:10:8:true LEADING:20 TRAILING:20 MINLEN:60). We aligned quality-filtered reads to the human reference genome (hg19) using bowtie2 with default parameters (version 2.3.2) and BMTagger (version 3.101). We kept only reads of which both paired ends failed to align in either tool. The average rates of host DNA contamination were 0.12% for fecal samples. As a final QC step, we removed duplicate reads using PRINSEQ-lite (version 0.20.4, parameters: -derep 1). We excluded one MS sample due to an extremely small number of QC passed reads and another MS sample due to the outlier in principal component analysis (PCA) of both phylogenetic data and gene abundance data (described below).

Taxonomic Annotation of Metagenome and Abundance Quantification

To improve both the efficiency and accuracy of taxonomic assignment, we selected the reference metagenomes of the Japanese population constructed by Nishijima *et al.* (Nishijima et al., 2016); 6,139 genomes from the National Center for Biotechnology Information and 10 genomes from in-house complete genome data constructed at Osaka University. Furthermore, we added newly reported genomes from the cultivated human gut bacteria projects (Almeida et al., 2019; Forster et al., 2019; Zou et al., 2019). After filtration to the genomes annotated to the species with more than 50 reference genomes, the taxonomic reference genome dataset consisted of 7,881 genomes. The filtered paired-end reads were aligned to the reference genome datasets using bowtie2 with default parameters (version 2.3.2). The average mapping rate was 88%. As for multiple-mapped reads, only the best possible alignment was selected by the alignment scores. The number of reads that mapped to each genome was divided by the length of the genome. The value of each genome was summed up by each sample, and the relative abundance of each clade was calculated at six levels (L2: phylum, L3: class, L4: order, L5: family, L6: genus, L7: species). For removing batch effects indicative of contaminants, we excluded clades that had been detected in neither of our previous metagenome cohorts (31 samples with average 29 Gb per sample and 96 samples with average 8.1 Gb, respectively) (Kishikawa et al., 2020a). At last we detected outlier samples by PCA.

Functional Annotation and Abundance Calculation

De novo assembly of the filtered paired-end reads into contigs was conducted using MEGAHIT (version 1.1.2, parameters: –min-contig-len 135). We predicted open reading frames (ORFs) on the contigs with the *ab initio* gene finder MetageneMark (version 3.38, parameters: -a -k -f G). Next, we annotated the ORF catalog with two protein databases, UniRef90 (Suzek et al., 2015) and Kyoto Encyclopedia of Genes and Genomes (KEGG; (Kanehisa and Goto, 2000). For the UniRef90 database, we selected prokaryotic, viral, and fungal data. For KEGG genes, we utilized a database of prokaryote KEGG genes and MGENES, a database of KEGG genes from metagenome samples annotated based on orthology, with a bit score >60. We aligned putative amino acid sequences translated from the ORF catalog against both databases with DIAMOND using BLASTP (v0.9.4.105, parameters: f 6 -b 15.0–k 1 -e 1e-6 –subject-cover 50). We identified 2,058,642 UniRef proteins and 1,248,480 KEGG genes. For quantification of ORF abundance, we mapped the filtered paired-end reads to the assembled contigs using bowtie2 with default parameters (version 2.3.2). To avoid the bias of the gene size, the ORF abundance was defined as the depth of each ORF's region of the ORF catalog according to the mapping result. As well as phylogenetic data, we excluded genes that had been detected in neither of our previous metagenome cohorts (Kishikawa et al., 2020a) and detected outlier samples by PCA. As mentioned above, one MS sample was excluded because it was the outlier of both phylogenetic data and gene abundance data.

Case-Control Association Test for Phylogenetic Data

We normalized the relative abundance profiles using the Box-Cox transformation function in the car R package (version 3.0.2), including log transformation. We removed clades detected (i) in

less than 20% of the samples, (ii) in no sample in either cases or controls, or (iii) with an average relative abundance of less than 0.001% of total abundance. After selection, we assessed 712 clades (10 phyla, 20 classes, 31 orders, 63 families, 166 genera, and 422 species). Case-control association tests were performed separately for each clade using the generalized linear model function in the R package glm2 (version 1.2.1). We adopted sex, age, and the top three principal components as covariates.

Case-Control Association Test for Gene Abundance Data

We converted each ORF abundance to annotated gene abundance for both UniRef90 protein and KEGG gene databases. We performed two steps of normalization. First, we adjusted the gene abundance by the sum of ORF abundance for each sample in order to correct the bias of the amount of sequence reads for each sample. Next, we applied a rank-based inverse normal transformation in order to correct the heterogeneity of each gene's abundance and distribution. We removed genes detected (i) in less than 20% of the samples or (ii) in no sample in either cases or controls. After gene selection, we assessed 219,715 genes annotated by the UniRef90 database and 222,606 genes annotated by the KEGG gene database. Case-control association tests were performed using the generalized linear model function in the R package glm2 (version 1.2.1). We adopted sex and age as covariates.

Metagenome Molecular Pathway Analysis

We performed gene set enrichment analysis using the R package clusterProfiler (version 3.8.1). Gene sets which contained over 30,000 genes or under 50 genes were excluded from the enrichment analysis. For case-control pathway association tests, genes annotated by the UniRef90 database were ranked based on their effect sizes of case-control gene association tests. The UniRef90 gene sets were composed according to Gene Ontology (GO) (Harris et al., 2004). Genes annotated by the KEGG gene database were ranked in the same way. The KEGG gene sets were defined according to the KEGG pathway.

Comparison of Gene Ontology Enrichment Analysis Results Between Multiple Sclerosis Metagenome and Host Genome-Wide Association Study

We assessed whether there were shared biological pathways between the gut metagenome and the human germline genome; we compared the GO enrichment data of the metagenome with that of the host GWAS in MS. For the host GWAS in MS, we obtained summary statistics from MS GWAS in the European population (n = 41,505) (International Multiple Sclerosis Genetics Consortium, 2019). We used Pascal with the summary statistics in order to determine GO enrichment of the germline in MS. We compared the P-values of GO shared between the GWAS data and metagenome data. We evaluated the overlap of the GO enrichment, by classifying the pathways based on the significance threshold of $P < 0.05$ or $P \geq 0.05$ and using Fisher's exact test.

Empirical Estimation of Metagenome-Wide Significance Threshold

We empirically estimated the statistical significance threshold separately for phylogenetic and gene case-control analyses, performing a phenotype permutation procedure (Kanai et al., 2016). We randomly simulated case-control phenotypes (×50,000 iterations) and calculated empirical null distributions of the minimum P-values (= P_{min}) in each iteration. We defined an empirical Bonferroni significance threshold, $-\log_{10}(P_{sig})$, as the 95th percentile of $-\log_{10}(P_{min})$ at a significance level of 0.05. We calculated $-\log_{10}(P_{sig})$ using the Harrell-Davis distribution-free quantile estimator (Harrell and Davis, 1982) and calculated a 95% confidence interval for $-\log_{10}(P_{sig})$ by a bootstrapping method in the R package Hmisc (version 4.1.1). To estimate the null distribution of the test statistics, we applied the same process used for minimum P-values to all the ranked P-values. We defined an empirical false discovery rate (FDR) threshold of 0.1 as the 90th percentile of $-\log_{10}$ P-values of each rank at a significance level of 0.1.

Multiple Sclerosis Case-Control Difference Between Alpha-Diversity and Beta-Diversity of the Metagenome

For calculating diversities, all samples were down-sampled at the same number of reads (n = 3,000,000). Alpha-diversity (within-sample diversity) was calculated based on gene abundance and six levels of phylogenetic relative abundance (L2–L7) for each sample according to the Shannon index. Statistical comparisons of Shannon index between MS cases and controls were assessed by Student's t-test. To quantify beta-diversity, non-metric multidimensional scaling on the Bray-Curtis dissimilarity was performed. For evaluating case-control differences in the dissimilarity, we performed permutational multivariate analysis of variance (PERMANOVA) (McArdle and Anderson, 2001) with 100,000 permutations using the R package vegan (version 2.5.4).

RESULTS

Shotgun Sequencing of MS Microbiome in the Japanese Population

We performed whole-genome shotgun sequencing of a total of 103 fecal DNA samples (26 individuals with MS and 77 healthy controls of Japanese ancestry; **Supplementary Table 1**), which passed stringent QC filters for the sequence reads and samples as described elsewhere (Kishikawa et al., 2020a). High-throughput whole-genome shotgun sequencing achieved relatively high read amounts per sample (average 7.3Gb), enabling robust implementation of a series of MWAS analyses.

Identification of Multiple Clades With Multiple Sclerosis Case-Control Discrepancy

After stringent QC for sequence reads and samples (**Supplementary Figure 1**), our MWAS assessed a total of 712

clades (10 phyla [L2], 20 classes [L3], 31 orders [L4], 63 families [L5], 166 genera [L6], and 422 species [L7]). Case-control phylogenetic association tests using a generalized linear regression model identified eight clades which conferred case-control discrepancy in their composition levels (empirically estimated FDR-q < 0.1; **Table 1**, **Figure 1A**). Of these, one exhibited increased abundances in the MS samples than in the controls (*Sutterella* sp.), whereas seven exhibited decreased abundances (*Erysipelatoclostridium* sp., *Gemella morbillorum*, *Granulicatella*, *Granulicatella adiacens*, *Gabonia*, *Gabonia massiliensis*, and *Carnobacteriaceae*; **Figure 1B**). Our analysis adjusted the confounding effects of sex and age by incorporating them as covariates. Furthermore, we confirmed that the results were independent of age and sex by conducting the stratified analyses (**Supplementary Table 2**). *Granulicatella adiacens* (L7), the genus *Granulicatella* (L6), and the family *Carnobacteriaceae* (L5), which were a series of the identical strain, showed significant correlations with age in MS samples, but not significant in control samples or all samples. We confirmed that the abundances of the clades in MS were less than those in controls across most generations (**Supplementary Figure 2**). To assess the correlation between the disease severity and the eight clade abundances, we divided the MS patients into two groups of severe MS (Expanded Disability Status Scale [EDSS] ≥ 4.5; n = 8) and mild MS (EDSS < 4.5; n = 18). We found no significant differences between the two groups, whereas severe MS exhibited 15.9 times more mean abundance of *Sutterella* sp. than that of mild MS (**Supplementary Table 3**).

As for the characteristics of the clades with case-control discrepancies, the genus *Erysipelatoclostridium* is mostly clostridium cluster XVIII reported to produce acetate, one of short-chain fatty acid (SCFA) (Narushima et al., 2014). SCFA plays a critical role in suppressing inflammation by inducing Tregs (Smith et al., 2013). SCFA was reported to ameliorate EAE (Haghikia et al., 2015) and modify blood-brain barrier permeability (Braniste et al., 2014). *Granulicatella adiacens*, the genus *Granulicatella*, and the family *Carnobacteriaceae* produce lactic acid (Siqueira and Rôças, 2006). An oral administration of the lactic acid bacterium ameliorated clinical EAE (Takata et al., 2011). *Gemella* was reported to reduce IL-12 levels in oral infections in mice (Ribeiro Sobrinho et al., 2002), while the role of *Gemella* in gut microbiome is unclear. IL-12 was one of the MS-associated genes found in MS GWAS (International Multiple Sclerosis Genetics Consortium, 2019). Thus, we detected the novel MS-associated clades related to the immune system. The clades could influence the pathology of MS. On the other hand, *Gabonia massiliensis* is a new species officially registered in 2015 (Lee et al., 2011; Mourembou et al., 2015). There have been few reports of the correlation of the clade and autoimmune diseases so far.

As for the previously reported MS-related clades of gut microbiome (Jangi et al., 2016; Cekanaviciute et al., 2017), we observed nominally significant associations in *Parabacteroides distasonis* (P = 0.01; **Supplementary Table 4**), providing rigorous evidence in our MWAS framework.

As illustrated in a phylogenetic tree indicating the case-control association results of multi-layered taxonomic levels (**Figure 1C**), five of the eight clades with case-control discrepancy belonged to species (L7, the most specific level). Since it was difficult to detect the species-level clades using classical 16S rRNA sequencing, our results clearly demonstrated the value of metagenome shotgun sequencing and the MWAS approach to identify disease-associated microbiome taxa. Each of the five species with case-control discrepancy belonged to different genera (L6) or families (L5), which was comparable to the previous reports that the majority of the disease-associated taxa belonged to relatively limited variety of genera or families (e.g., *Prevotella* families in rheumatoid arthritis [Maeda et al., 2016; Kishikawa et al., 2020a]). Our results should empirically propose polytaxonomic architecture of MS microbiome rather than monotaxonomic one, which represents contribution of relatively wider ranges of taxa with moderate effects.

High Abundance of an ABC Transporter-Related Gene in Multiple Sclerosis Metagenome

The MWAS framework can quantitatively assess case-control discrepancy of gene abundances in metagenome, which deserves discovery of novel therapeutic targets. To this end, we performed the following procedures: (i) *de novo* assembly, (ii) prediction of ORFs, and (iii) calculating the ORF abundances by mapping the reads to the assembled contigs. After QC for the genes, we obtained 219,715 and 222,606 genes annotated by the UniRef90 and KEGG databases, respectively. Case-control gene association tests utilizing a generalized linear regression model found that two genes registered at KEGG increased in the MS samples compared to the controls (FDR-q < 0.1, Clo1100_2356 and Mahau_1952; **Figure 2** and **Table 2**). Clo1100_2356 is

TABLE 1 | Clades in gut microbiome with MS case-control discrepancy.

Microbe	Level	Effect size	*P*-value
Erysipelatoclostridium sp.	Species (L7)	−1.09	6.9×10^{-4}
Gemella morbillorum	Species (L7)	−0.95	0.0012
Granulicatella	Genus (L6)	−0.77	0.0031
Granulicatella adiacens	Species (L7)	−0.79	0.0032
Gabonia	Genus (L6)	−1.53	0.0036
Gabonia massiliensis	Species (L7)	−1.53	0.0036
Sutterella sp.	Species (L7)	2.73	0.0038
Carnobacteriaceae	Family (L5)	−0.69	0.0039

MS, multiple sclerosis.

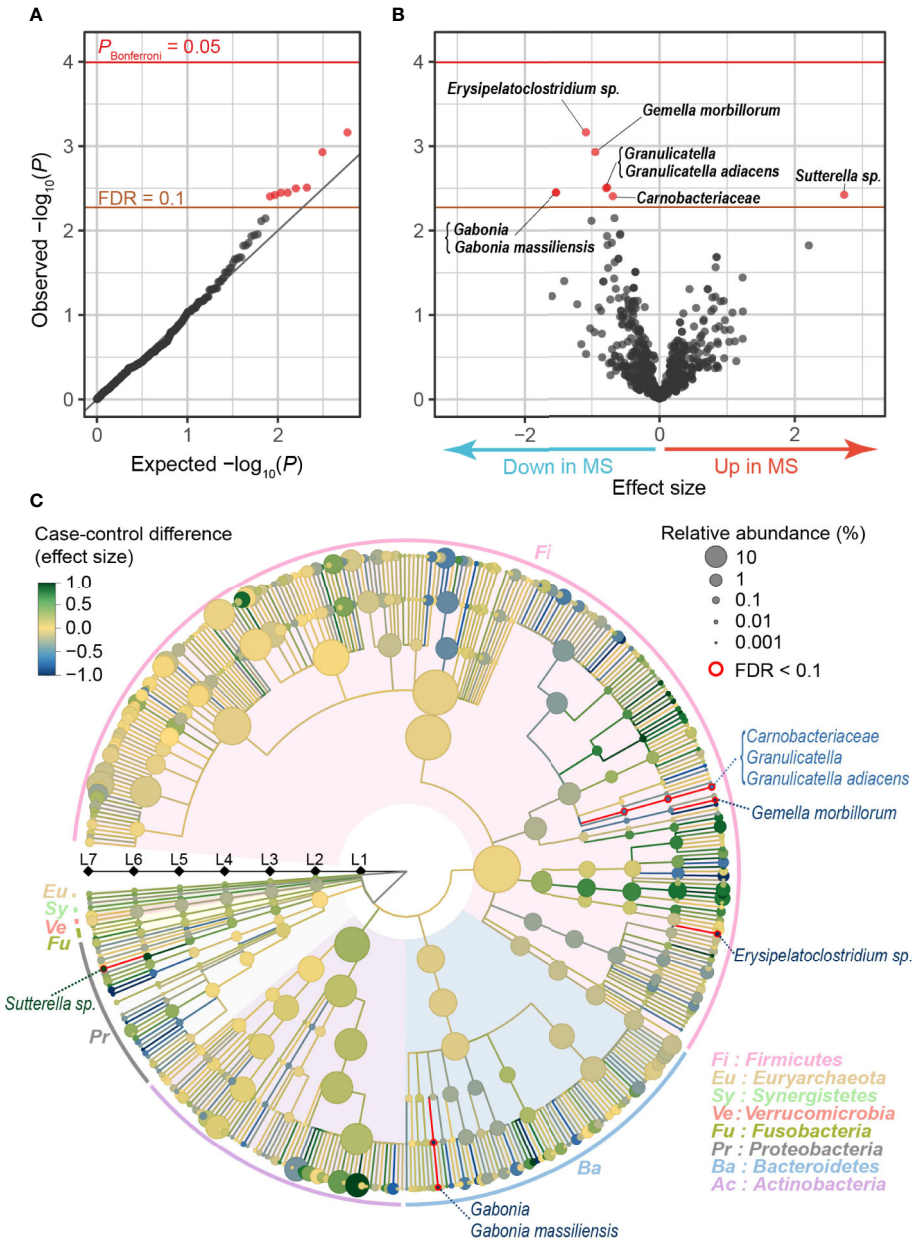

FIGURE 1 | MWAS results of MS case-control phylogenetic association tests. **(A)** A quantile-quantile plot of the MWAS *P*-values of the clades. The *x*-axis indicates empirically estimated median -$\log_{10}$ *P*-values. The *y*-axis indicates observed -$\log_{10}$ *P*-values. The diagonal gray line represents $y = x$, which corresponds to the null hypothesis. The horizontal red line indicates the empirical Bonferroni-corrected threshold ($\alpha = 0.05$), and the brown line indicates the empirically estimated (FDR-$q = 0.1$). Clades with FDR-$q < 0.1$ are plotted as red dots, and other clades as black dots. **(B)** A volcano plot. The *x*-axis indicates effect sizes of generalized linear model. The *y*-axis, horizontal lines, and dot colors are the same as in panel **(A)**. **(C)** Phylogenetic tree. Levels L2–L7 are from the inside layer to the outside layer. The size and color of dots represent relative abundance and effect sizes, respectively. The five clades with suggestive case-control associations (FDR-$q < 0.1$) are outlined in red. FDR, false discovery rate; MWAS, metagenome-wide association study; MS, multiple sclerosis.

categorized in glycerol-3-phosphate dehydrogenase, and Mahau_1952 is defined as ABC transporter related protein. It has been suggested that ABC transporter of microbiome affects the etiology of neuromyelitis optica (NMO), an MS-related disease. T cells specific for aquaporin 4, the major antigen of NMO, cross-react with the homologous ABC transporter peptide of *Clostridium perfringens*, suggesting that molecular mimicry against microbial antigens could induce the autoimmune response in NMO (Varrin-Doyer et al., 2012). Although the specific antibody of MS has never been detected, the increased abundance of ABC transporter related gene in MS metagenome implies a similar immune mechanism in MS.

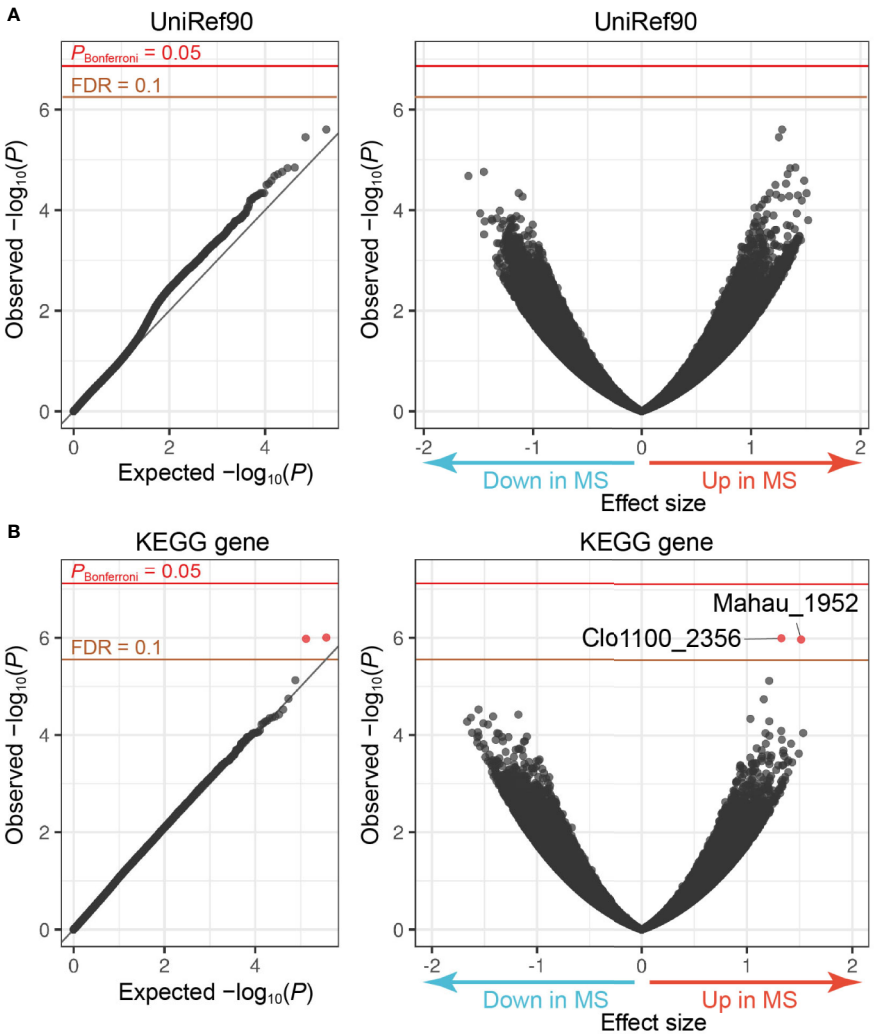

FIGURE 2 | MWAS results of MS case-control gene association tests. **(A)** A quantile-quantile plot (left) and a volcano plot (right) of the MWAS *P*-values of genes based on the UniRef90 protein database. **(B)** A quantile-quantile plot (left) and a volcano plot (right) of genes based on the KEGG gene database. In the quantile-quantile plots, the *x*-axis indicates empirically estimated median -$\log_{10}$ *P*-values. In the volcano plot, the *x*-axis indicates beta of generalized linear model as effect sizes. The *y*-axis in both plots indicates observed -$\log_{10}$ *P*-values. The diagonal gray line represents $y = x$, which corresponds to the null hypothesis. The horizontal red line indicates the empirical Bonferroni-corrected threshold ($\alpha = 0.05$), and the brown line indicates the empirically estimated (FDR-q = 0.1). Genes with FDR-q < 0.1 are plotted as red dots, and other genes as black dots.

We evaluated the effects of sex and age on the abundances of these two genes, founding no significant effects (**Supplementary Table 2**). Although *P*-value of sex differences of Mahau_1952 in MS samples was less than 0.05, the direction of the increase or decrease was opposite to the result of case-control association test; the abundances in males were more than those in females. There existed no significant effects of EDSS as well (**Supplementary Table 3**).

Alteration of Pathways Related to Lipopolysaccharide in Multiple Sclerosis

We then performed gene set enrichment analysis to conduct case-control pathway association tests using the results of the gene analysis of our MWAS. We found significant associations for 13 GO terms and 2 KEGG pathways that satisfied the Bonferroni's correction (**Figures 3A, B**, and **Table 3**). One of the GO terms with

TABLE 2 | Metagenome genes with MS case-control discrepancy.

KEGG gene	Effect size	*P*-value	Definition	KEGG Orthology
Clo1100_2356	1.33	9.9×10^{-7}	putative dehydrogenase	glpA, glpD; glycerol-3-phosphate dehydrogenase
Mahau_1952	1.54	1.1×10^{-6}	ABC transporter related protein	ABC-2.A; ABC-2 type transport system ATP-binding protein

KEGG, Kyoto Encyclopedia of Genes and Genomes; MS, multiple sclerosis.

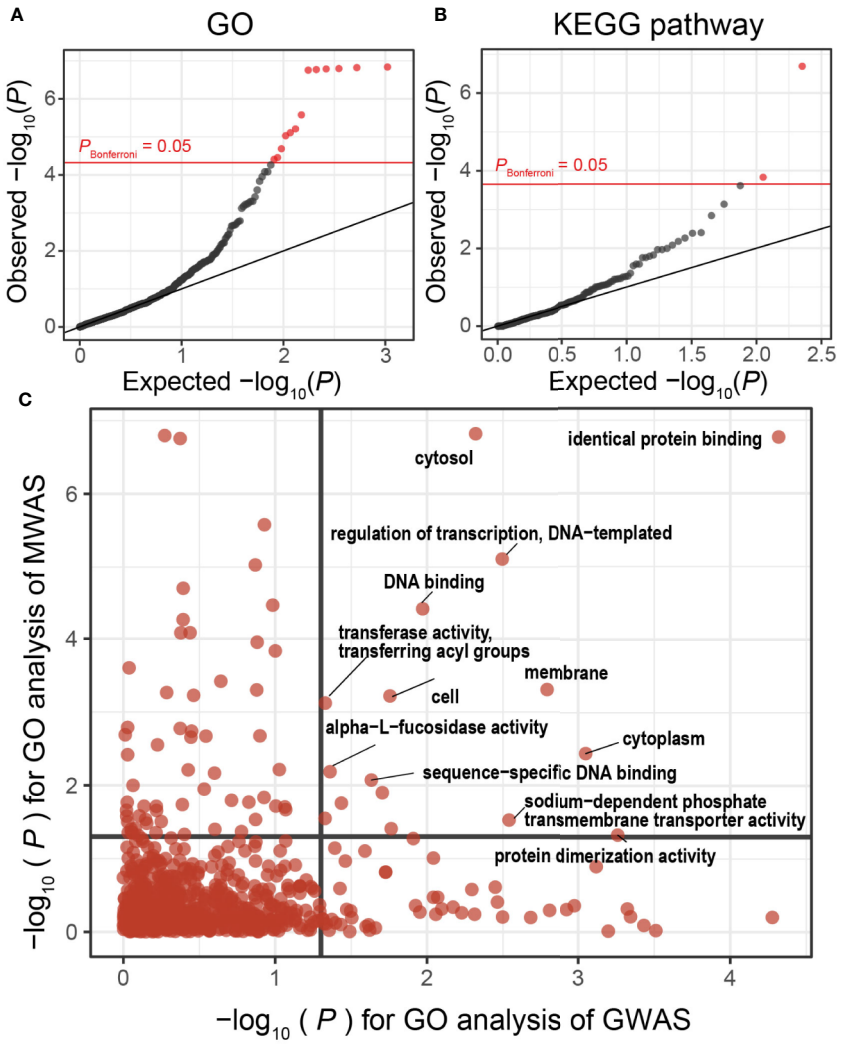

FIGURE 3 | MWAS results of MS case-control pathway association tests. **(A)** A quantile-quantile plot of the MWAS *P*-values of enrichment analyses based on GO terms. GO terms with *P*-values less than Bonferroni thresholds are plotted as red dots, and the other clades as black dots. **(B)** A quantile-quantile plot of the MWAS *P*-values of enrichment analyses based on KEGG pathways. **(C)** Comparison of *P*-values of GO enrichment analyses between the MS MWAS and GWAS data. The *x*-axis indicates the *P*-values of the GWAS. The *y*-axis indicates the *P*-values of the MWAS. The horizontal and vertical black lines indicate *P*-value of 0.05. The overlap of the GO enrichment was evaluated by classifying the GO terms based on the significance threshold of $P < 0.05$ or $P \geq 0.05$ and using Fisher's exact test. GO, Gene Ontology; GWAS, genome-wide association study; KEGG, Kyoto Encyclopedia of Genes and Genomes; MWAS, metagenome-wide association study; MS, Multiple sclerosis.

significant enrichments was cell outer membrane ($P = 1.5 \times 10^{-7}$, GO:0009279). Major component of the outer membrane of gram-negative bacteria was lipopolysaccharides (LPS), which prompts activation of cell and secretion of inflammatory cytokines by engaging Toll-like receptor (Stevens et al., 2015). As for MS, LPS has been reported to induce and worsen experimental autoimmune encephalomyelitis (Nogai et al., 2005). We also found that KEGG pathway of lipopolysaccharide biosynthesis ($P = 2.1 \times 10^{-7}$, ko00540) was significantly altered. Although the involvement of LPS in MS is already known, this study is first to robustly demonstrate the involvement using functional analysis of shotgun sequencing.

Evidence of Shared Molecular Pathways Between the Metagenome and Host Genome in Multiple Sclerosis

Disentanglement of interaction between host genomics and metagenome is a key concept towards elucidation of disease etiology. While human host genes and gene products of microbiome were categorized as different omics layers, one can directly connect them though *in silico* trans-omics connection by projection to the molecular pathways (Kishikawa et al., 2020a). To this end, we evaluated whether disease-associated molecular pathways represented as the GO terms were shared between the gut metagenome and the host germline genome. In addition to

TABLE 3 | Pathways with case-control discrepancy in MS MWAS.

GO term	Set size	*P*-value	Name
GO:0009279	572	1.5×10^{-7}	cell outer membrane
GO:0005829	408	1.5×10^{-7}	cytosol
GO:0015562	228	1.6×10^{-7}	efflux transmembrane transporter activity
GO:0030288	176	1.6×10^{-7}	outer membrane-bounded periplasmic space
GO:0042802	102	1.7×10^{-7}	identical protein binding
GO:0006974	54	1.8×10^{-7}	cellular response to DNA damage stimulus
GO:0000272	85	2.6×10^{-6}	polysaccharide catabolic process
GO:0042597	190	6.2×10^{-6}	periplasmic space
GO:0006355	4053	7.8×10^{-6}	regulation of transcription, DNA-templated
GO:0005886	9545	9.4×10^{-6}	plasma membrane
GO:0004853	69	2.1×10^{-5}	uroporphyrinogen decarboxylase activity
GO:0008422	177	3.5×10^{-5}	beta-glucosidase activity
GO:0003677	20242	3.9×10^{-5}	DNA binding
KEGG pathway	**Set size**	***P*-value**	**Name**
ko00540	318	2.1×10^{-7}	Lipopolysaccharide biosynthesis
ko00790	794	1.5×10^{-4}	Folate biosynthesis

GO, gene ontology; MS, multiple sclerosis; MWAS, metagenome-wide association study.

the GO enrichments of the MWAS mentioned above, we estimated GO enrichments in the association signals observed in the previously conducted MS GWAS data (n = 41,505) (International Multiple Sclerosis Genetics Consortium, 2019). We quantified MWAS-GWAS interaction by comparing the *P*-values of the GO terms shared between the MS MWAS data and the MS GWAS data (**Figure 3C**). Multiple GO terms showed significant enrichments between the metagenomes and host genomes of MS ($P_{GO} < 0.05$ in both). Of these, the identical protein binding term demonstrated notably significant enrichments ($P_{GO} = 1.7 \times 10^{-7}$ and 4.8×10^{-5} in MWAS and GWAS, respectively), suggesting its prominent roles in MS pathophysiology. We observed a significant correlation between *P*-values of GO terms ($P_{Fisher} = 1.7 \times 10^{-4}$). This MWAS-GWAS interaction provided empirical evidence of biological link between the germline genome and metagenome in MS pathology.

No Apparent Discrepancies in Metagenome Diversity Between Multiple Sclerosis Cases and Controls

There have been studies assessing whether taxonomic diversities of gut microbiome differ between MS cases and controls or not (Miyake et al., 2015; Chen et al., 2016; Jangi et al., 2016; Berer et al., 2017; Cekanaviciute et al., 2017), along with the discussions on how to locate dysbiosis in the pathogenicity of MS. We thus assessed alpha- and beta-diversity in both of the phylogenetic data (phylogenetic relative abundance of six levels [L2–L7]) and functional data (gene abundance based on the UniRef90 protein and KEGG gene databases). Not any levels of phylogenetic data showed significant case-control differences in alpha-diversity based on the Shannon index ($P > 0.05$; **Figure 4A**). The gene abundance data did not show significant differences either ($P > 0.05$; **Figure 4B**). Similarly, no significant differences in beta-diversity were found in any case-control comparisons ($P > 0.05$; **Figures 4C, D**). Overall, there exist no apparent discrepancies in metagenome diversity between MS cases and controls.

DISCUSSION

In this study, we conducted a comprehensive MWAS of MS in the Japanese population, by utilizing whole-genome shotgun sequencing. Our study identified the following novel features of the MS gut metagenome: (i) Eight clades, mostly related to immune systems, showed discrepancies in the case-control comparison. (ii) The abundances of two genes increased in the MS metagenome, including the ABC transporter related gene which could induce autoimmune response through molecular mimicry. (iii) Molecular pathways related to LPS prompting secretion of inflammatory cytokines were altered in the MS metagenome. (iv) Significant interaction of the pathways between the metagenome (MWAS) and the host genome (GWAS) was identified. (v) No apparent discrepancies in metagenome diversities were found between the MS cases and controls. Our study greatly exploited the benefits of shotgun sequencing, since these characteristics would be difficult to find by using the classical method of 16S rRNA.

Our study has an advantage in high resolution analysis focusing the species level (L7). Of the eight clades with MS case-control discrepancy in our study, the genus *Sutterella* has been reported to increase in the gut microbiome of MS patients under treatment compared to healthy controls, while decreasing in untreated MS patients (Jangi et al., 2016). In our study, there existed a higher relative abundance of *Sutterella* sp. in the MS patients under treatment than in the untreated MS patients (**Supplementary Table 3**). When stratifying the MS patients by both severity of MS and treatment status, it was suggested that they influenced the abundance of *Sutterella* sp. independently (**Supplementary Figure 3**). Thus, the increased level of *Sutterella* sp. in our study could be an effect of medication because most patients (90%) were under treatment. The other seven clades were not influenced by treatment status.

Our MWAS on genes and molecular pathways successfully found the novel functional aspects of the MS gut metagenome. In the taxonomic assignment of Clo1100_2356, The Clo1100_2356 sequences in our metagenome data were mainly linked to the taxonomic reference genomes of *Firmicutes bacterium* and

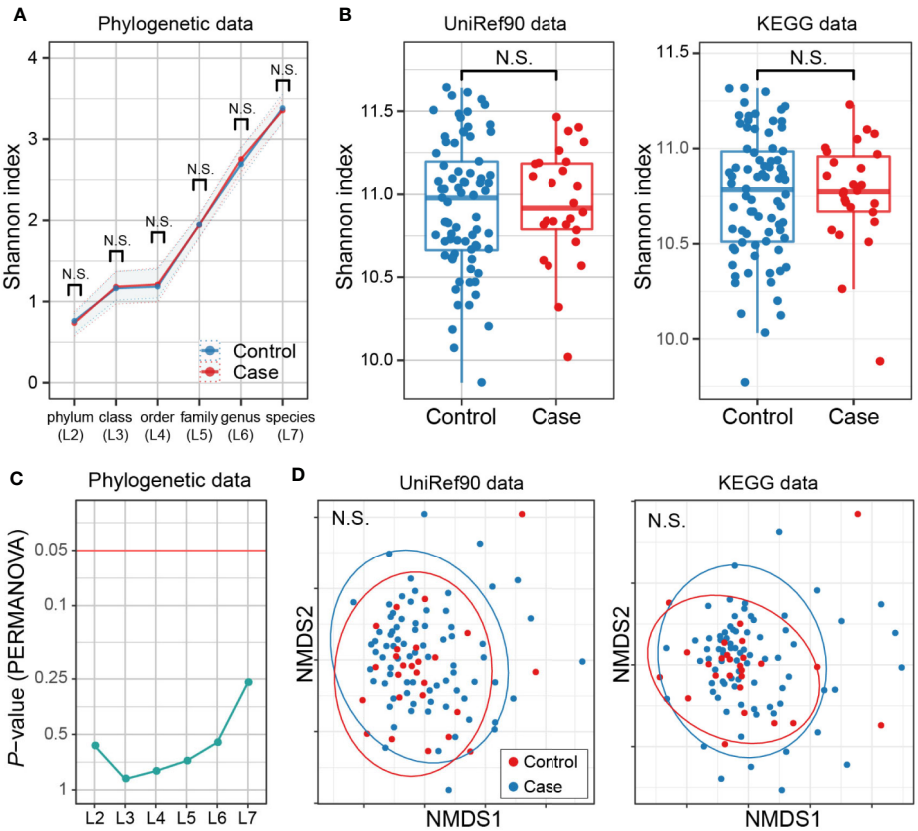

FIGURE 4 | MS case-control comparison of microbial diversities. **(A)** Alpha diversities of the phylogenetic relative abundance data for six levels. Welch's t-test of Shannon index between MS cases and controls showed no significant difference at any level. **(B)** Alpha diversities of the gene abundance data of the UniRef90 protein and KEGG gene databases. No significant case-control difference was found. **(C)** Beta diversities of phylogenetic relative abundance data at six levels. PERMANOVA based on Bray-Curtis dissimilarities found no significant differences among levels for either sequencing group with Bonferroni correction. **(D)** Beta diversities of the gene abundance of the UniRef90 protein database. No significant case-control difference was found. KEGG, Kyoto Encyclopedia of Genes and Genomes; NMDS, non-metric multidimensional scaling; PERMANOVA, permutational multivariate analysis of variance; MS, Multiple sclerosis.

Ruminococcus sp., while the source organism is registered as *Clostridium* sp. in the KEGG database. The Mahau_1952 sequences in our metagenome data were mainly linked to the taxonomic reference genomes of *Dorea formiciigenerans* and *Lachnospiraceae bacterium*. We found no direct association between our findings of phylogenetic analysis and gene analysis.

The studies focusing on the interaction between the metagenome and host genome in human complex diseases have become an interesting topic in the field of microbiome (Imhann et al., 2018; Asquith et al., 2019). Our study is the first to demonstrate the MWAS-GWAS interaction of the molecular pathways in the MS gut metagenome. Our results should warrant further studies to elucidate functional connection between MS metagenome and host genome.

Ma et al. performed a comprehensive analysis of the microbial diversity in microbiome-associated diseases (not including MS) and indicated that there were no significant differences in most diseases relative to controls (Ma et al., 2019). Our results supported this finding for MS in the aspects of both taxonomic and functional gene diversity. Thus, MS gut microbiome is characterized by combination of specific disease-associated clades, genes, and molecular pathways rather than overall dysbiosis.

In this study, age and sex between case and control were not completely matched. However, we corrected for their bias by analyzing with incorporation of sex and age as covariates. We also confirmed that age and sex did not significantly affect the genes and clades with MS case-control discrepancies. Furthermore, Odamaki et al. examined changes in the gut microbiome composition of the Japanese population with age in a wide range of age groups from newborn to centenarian (Odamaki et al., 2016). They demonstrated that the microbiome composition remained relatively stable between the 20s and 60s, the relevant age group in our study. Thus, we may detect fewer findings than expected by adjusting confounding effects, but the results of this study are robust regardless of age and sex.

CONCLUSIONS

In conclusion, our shotgun sequencing-based comprehensive MWAS revealed novel characteristics of the MS gut microbiome and the interaction between the gut metagenome and host genome. Our study will provide useful resources for future functional investigations to further elucidate details of the microbiome's role in MS etiology.

DATA AVAILABILITY STATEMENT

The whole-genome shotgun sequencing data are deposited in National Bioscience Database Center (NBDC) Human Database (http://humandbs. biosciencedbc. jp/) with the accession number of hum0197. The data are available upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committees of Osaka University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TK and YO designed the study, conducted the data analysis, and wrote the manuscript. TK, DM, YMat, TN, YMae, and SN conducted the experiments. TK, KO, AH, MK, KS, KY, TM, SN, HI, and TO collected the samples. HI, HM, TO, and YO supervised the study. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (19H01021 and 20K21834), the Japan Agency for Medical Research and Development (AMED; JP20ek0109413, JP20km0405211, JP20ek0410075, JP20gm4010006, and JP20km0405217), Takeda Science Foundation, Bioinformatics Initiative of Osaka University Graduate School of Medicine, Grant Program for Next Generation Principal Investigators at Immunology Frontier Research Center (WPI-IFReC), Osaka University.

ACKNOWLEDGMENT

This research was supported by the Clinical Investigator's Research Project of the Osaka University Graduate School of Medicine.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.585973/full#supplementary-material

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