Reductions in anti-inflammatory gut bacteria are associated with depression in a sample of young adults

Richard T. Liu[a],[1],[*], Aislinn D. Rowan-Nash[b],[1], Ana E. Sheehan[c], Rachel F.L. Walsh[d], Christina M. Sanzari[a], Benjamin J. Korry[b], Peter Belenky[b]

[a] Department of Psychiatry and Human Behavior, Alpert Medical School of Brown University, Providence, RI, USA
[b] Department of Molecular Microbiology and Immunology, Brown University, Providence, RI, USA
[c] Department of Psychological and Brain Sciences, University of Delaware, Newark, DE, USA
[d] Department of Psychology, Temple University, Philadelphia, PA, USA

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ABSTRACT

We assessed the gut microbiota of 90 American young adults, comparing 43 participants with major depressive disorder (MDD) and 47 healthy controls, and found that the MDD subjects had significantly different gut microbiota compared to the healthy controls at multiple taxonomic levels. At the phylum level, participants with MDD had lower levels of Firmicutes and higher levels of Bacteroidetes, with similar trends in the at the class (Clostridia and Bacteroidia) and order (Clostridiales and Bacteroidales) levels. At the genus level, the MDD group had lower levels of *Faecalibacterium* and other related members of the family *Ruminococcaceae*, which was also reduced relative to healthy controls. Additionally, the class Gammaproteobacteria and genus *Flavonifractor* were enriched in participants with MDD. Accordingly, predicted functional differences between the two groups include a reduced abundance of short-chain fatty acid production pathways in the MDD group. We also demonstrated that the magnitude of taxonomic changes was associated with the severity of depressive symptoms in many cases, and that most changes were present regardless of whether depressed participants were taking psychotropic medications. Overall, our results support a link between MDD and lower levels of anti-inflammatory, butyrate-producing bacteria, and may support a connection between the gut microbiota and the chronic, low-grade inflammation often observed in MDD patients.

1. Introduction

There is increasing recognition of the fact that the gut microbiota is associated with a wide range of health conditions and disease states in humans. Alterations to gut microbiome composition or function have been linked to gastrointestinal disorders (Kostic et al., 2014; Ahn et al., 2013), autoimmune disorders (He et al., 2016; Scher et al., 2016; Brown et al., 2011; de Paiva et al., 2016; Huang et al., 2019; Kasselman et al., 2018), and metabolic and cardiovascular disease (Kasselman et al., 2018; Arora and Backhed, 2016; Jie et al., 2017). Perhaps most surprisingly, given the physiological distance and the presence of the blood–brain barrier, the gut microbiota has also been implicated in psychiatric disorders or syndromes, including anxiety disorders (Foster and McVey Neufeld, 2013; Jiang et al., 2018; Chen et al., 2019), bipolar disorder (Evans et al., 2017; Coello et al., 2019; Dickerson et al., 2017; Nguyen et al., 2018), and major depressive disorder (MDD) (Foster and

McVey Neufeld, 2013; Jiang et al., 2015; Huang et al., 2018; Zheng et al., 2016; Naseribafrouei et al., 2014; Chen et al., 2018; Valles-Colomer et al., 2019; Lai et al., 2019; Rong et al., 2019; Lin et al., 2017).

Links between the gut microbiota and the brain are likely mediated in part through the gut-brain axis (GBA), a proposed series of complex communication pathways between the gastrointestinal tract and the central nervous system (Sharon et al., 2016; Carabotti et al., 2015; O'Mahony et al., 2015; Kelly et al., 2016; Foster et al., 2017; Mayer et al., 2015; Dinan et al., 2015). The GBA includes neural, immune, endocrine, and metabolic pathways involved in the regulation of hunger and satiety, stress, immunity, and intestinal motility; the gut microbiota are believed to play a direct role in some of these. In particular, microbes or their metabolites such as short-chain fatty acids (SCFA) can stimulate afferent inputs to the vagus nerve (Bonaz et al., 2019; Forsythe et al., 2014; Caspani et al., 2019), induce

enteroendocrine cells to produce neuropeptides and activate afferent nerve pathways (Raybould, 2010; Sarkar et al., 2016), promote normal stress responses and hypothalamic–pituitaryadrenal (HPA) axis development and function (O'Mahony et al., 2009; Sudo et al., 2004), and participate in local neurotransmitter production and systemic regulation via tryptophan metabolism or direct secretion (O'Mahony et al., 2015; Wikoff et al., 2009; Yano et al., 2015; Barrett et al., 2012). In the other direction, the vagus nerve can promote anti-inflammatory responses and decrease intestinal permeability (Bonaz et al., 2019; Forsythe et al., 2014; Borovikova et al., 2000; Johnston and Webster, 2009), stress-induced glucocorticoid induction through the HPA axis can lead to microbial changes and increased gut barrier permeability (Park et al., 2013; de Punder and Pruimboom, 2015; Vicario et al., 2010; Kelly et al., 2015; Rodino-Janeiro et al., 2015; Moussaoui et al., 2014; Soderholm et al., 2002), and the central nervous system can influence the gut environment through release of signalling molecules, changes to mucus secretion, and regulation of intestinal motility (Carabotti et al., 2015; Mayer et al., 2015; Rhee et al., 2009; Martin et al., 2018; Mayer, 2011).

In terms of depression, links to the microbiome have been established in both human and animal models (Huang et al., 2019; Dinan and Cryan, 2013; Evrensel and Ceylan, 2015; Lach et al., 2018). In one particularly compelling case, fecal transplants from humans with MDD resulted in the development of depressive symptoms in a germ-free mouse model (Zheng et al., 2016). Similarly, fecal transplants from depressed human subjects into a germ-free rat model induced development of depressive symptoms, including anhedonia and anxiety, in addition to changes in tryptophan metabolism (Kelly et al., 2016). Additionally, certain microbes including *Faecalibacterium prausnitzii*, *Lactobacillus* spp., and *Bifidobacterium* spp. have been found to ameliorate the onset of anxiety and depressive symptoms that rodents develop when subjected to chronic unpredictable mild stress, maternal separation, or chemically-induced colitis (Hao et al., 2019; Bravo et al., 2011; Bercik et al., 2011). Finally, recent work has demonstrated that treatment of rats bred for high-anxiety behavior with the antimicrobial minocycline altered the gut microbiota while also alleviating depressive symptoms, supporting previous work suggesting that minocycline may have merit as an adjunct treatment alongside antidepressants (Schmidtner et al., 2019; Husain et al., 2017; Dean et al., 2017).

In humans, studies have consistently indicated that the gut microbiota of adults with MDD are different from those of their healthy counterparts, although specific differences have varied between studies. Some studies have found that the phylum Bacteroidetes is underrepresented in subjects with depression, while Firmicutes are overrepresented (Zheng et al., 2016; Naseribafrouei et al., 2014; Lai et al., 2019; Rong et al., 2019; Lin et al., 2017), although other studies have found the opposite trend (Jiang et al., 2015; Huang et al., 2018). Multiple studies have linked higher abundance of the genera *Alistipes, Oscillibacter,* and *Flavonifractor* and the family *Enterobacteriaceae* to MDD and low quality of life scores (Jiang et al., 2015; Zheng et al., 2016; Naseribafrouei et al., 2014; Chen et al., 2018; Valles-Colomer et al., 2019; Lai et al., 2019; Rong et al., 2019), while *Faecalibacterium*, *Dialister, Coprococcus*, and *Prevotella* have been found to be lower in subjects with depression and/or low quality of life scores (Jiang et al., 2015; Zheng et al., 2016; Chen et al., 2018; Valles-Colomer et al., 2019; Kelly et al., 2016). *Faecalibacterium* has even been found to negatively correlate with depression severity (Jiang et al., 2015) and this species has also been found to be negatively associated with both bipolar disorder and generalized anxiety disorder (Jiang et al., 2018; Evans et al., 2017).

A potential link between the gut microbiota and MDD is the low-grade, chronic inflammation that has previously been observed in a substantial proportion of depressed individuals (Felger and Lotrich, 2013; Amodeo and Fagiolini, 2017; Miller and Raison, 2016). Significant subsets of depressed subjects have been associated with higher levels of circulating inflammatory cytokines, particularly IL-6 and TNF-α (Dowlati et al., 2010; Kohler et al., 2017; Haapakoski et al., 2015; Liu et al., 2012; Goldsmith et al., 2016; Himmerich et al., 2019), in addition to hypercortisolism and dysregulation of the HPA axis (Burke et al., 2005; Carroll et al., 2007; Parker et al., 2003). Furthermore, a few studies have demonstrated that combining antidepressants with anti-inflammatory drugs improved response rates (Mendlewicz et al., 2006; Muller et al., 2006), and inflammasome signalling has been linked to induction of anxiety and depressive behaviors in mice (Wong et al., 2016; Zhang et al., 2015; Alcocer-Gomez et al., 2016; Iwata et al., 2013; Kaufmann et al., 2017). Human patients with chronic inflammatory illnesses have higher levels of depression than the general population (Barnard et al., 2006; Dickens et al., 2002; Figueiredo-Braga et al., 2018; Byrne et al., 2017; Caneo et al., 2016), and administration of inflammatory cytokines or immune-provoking stimuli such as lipopolysaccharide (LPS) leads to the development of "sickness behavior" and depressive symptoms in both animal models and human patients (Dantzer, 2004; Raison and Miller, 2013; Miyaoka et al., 1999; O'Connor et al., 2009; Ohgi et al., 2013; Zhang et al., 2014; Fu et al., 2010; Anisman et al., 2005; Schiepers et al., 2005). Mechanistically, inflammatory cytokines may increase blood–brain barrier permeability and in some cases cross it (Schiepers et al., 2005; Rochfort et al., 2014; Farkas et al., 1998; de Vries et al., 1996; Plotkin et al., 1996; Watkins et al., 1995; Miller et al., 2009), activate vagus nerve afferents (Johnston and Webster, 2009; Watkins et al., 1995; Miller et al., 2009; Pavlov and Tracey, 2012; Steinberg et al., 2016; Zanos et al., 2018), impact neurotransmitter levels in the brain (Watkins et al., 1995; Miller et al., 2009; Galic et al., 2012; De Simoni and Imeri, 1998; Miller et al., 2013), contribute to hyperactive dysregulation of the HPA axis (Gadek-Michalska et al., 2013; Dantzer et al., 1999; Zunszain et al., 2011; Humphreys et al., 2006; Pace et al., 2007; Miller et al., 1999), and affect serotonergic neurotransmission by promoting enzymatic metabolism of the precursor tryptophan (Schiepers et al., 2005; Capuron et al., 2002; Dantzer et al., 2011; Maes et al., 1997; Myint and Kim, 2003; Riedel et al., 2002; Heyes et al., 1992).

The gut microbiota may contribute to such an effect through their capacity to either promote or protect against inflammation. For example, loss of bacteria that produce the anti-inflammatory, barrier-strengthening molecule butyrate, such as *Faecalibacterium* or *Coprococcus*, could lead to a loss of protection against epithelial inflammation and gut barrier disruption. Combined with increases in LPS-producing bacterial groups such as Proteobacteria or potentially pro-inflammatory species such as *Flavonifractor*, this could lead to increased translocation of immunogenic bacterial products and activation of low-grade systemic inflammation. In fact, studies have found that depressed subjects have increased levels of bacterial DNA in circulation and increased antibody responses to LPS (Maes et al., 2008; Maes et al., 2012; Maes et al., 2013; Keri et al., 2014; Berk et al., 2013).

In this study, we analyzed the gut microbiota of young adults with major depressive disorder and healthy controls. We hypothesized that we would observe potential signatures of inflammation, including either reductions in protective, butyrate-producing bacterial taxa or increases in pro-inflammatory taxa. Our study differs from previous studies in terms of demographics, as most previous studies have been performed on subjects of Chinese heritage (Chen et al., 2018; Jiang et al., 2015; Huang et al., 2018; Zheng et al., 2016; Lai et al., 2019; Rong et al., 2019; Lin et al., 2017) and a few have examined European subjects (Naseribafrouei et al., 2014; Valles-Colomer et al., 2019; Kelly et al., 2016), but to our knowledge none have so far assessed differences in American subjects. Furthermore, most previous studies have examined older age groups, with age ranges in the thirties and forties, while we utilized a sample of participants aged 18–25. Given previous work demonstrating that age, ethnic background, and geographic location can have significant impacts on the composition and function of the microbiota (Costello et al., 2009; Gupta et al., 2017; Yatsunenko et al., 2012; Odamaki et al., 2016; Yadav et al., 2016; Nam et al., 2011; Vangay et al., 2018), this study provides an important new perspective

on the gut microbiome in the context of MDD. Finally, our study analyzed a large sample which included both a notable subset of MDD participants who were not taking psychotropic medications as well as MDD subjects with a range of symptom severities. This allowed us to assess the potential contributions of these characteristics to changes in the gut microbiota observed in participants with MDD. Overall, we observed that the MDD subjects exhibited lower levels of potentially protective taxa, including *Faecalibacterium* and *Subdoligranulum,* and higher levels of potentially pro-inflammatory taxa, including *Flavonifractor* and Gammaproteobacteria; furthermore, many of these changes track with symptom severity.

2. Methods

2.1. Participants and sample collection procedures

Young adults were recruited from the community and local psychiatric clinics through flyers and social media advertisements between May 2018 and July 2019. All participants were recruited from the Rhode Island community and psychiatric clinics in Providence, RI. To participate in the study, prospective participants were required to be 18–25 years old, meet eligibility criteria for either the MDD group or the healthy control group, and not be subject to any exclusion criteria. To be eligible for the healthy control group, subjects were required to have a Patient-Reported Outcomes Measurement Information System (PROMIS) Depression score <13, no lifetime history of major depression, no lifetime history of suicidal ideation, suicide attempts, or nonsuicidal self-injury as assessed by the Columbia-Suicide Severity Rating Scale (C-SSRS) and Self-Injurious Thoughts and Behavior Interview (SITBI). To be eligible for the MDD group, participants needed to meet diagnostic criteria for a current major depressive episode and have PROMIS Depression scores >21. Individuals were excluded if they had smoked cigarettes or cigars in the past 12 months, were vegan, had gastrointestinal illness in the past six months, had diarrhea in the past two weeks, used anti-diarrhea medication in the past six weeks, or used antibiotics in the past three months.

Prospective participants first completed an online screener which included the PROMIS Depression scale and questions regarding the exclusion criteria. Those who remained potentially eligible based on the online screener then completed a phone screener, in which they answered the Structured Clinical Interview for DSM-5 Disorders (SCID-V), C-SSRS, and SITBI. Those who were eligible were invited to attend a one-hour in-person assessment, at which they were re-administered the PROMIS Depression questionnaire and were asked to provide a stool sample using OMNIgene•GUT stool collection kits (DNA Genotek). Whole blood was drawn by licensed phlebotomists, allowed to clot for 15 min at room temperature, centrifuged for 10 min, and immediately aliquoted and stored at −80 °C until use. Data about psychotropic medication usage was also collected.

The final sample consisted of 90 participants (see Table 1 and Fig. 1). The healthy control group (n = 47) had a mean age of 21.7 ± 2.1, mean PROMIS Depression scores of 9.3 ± 1.4, and was 72.3% female, 76.7% white, and 14.0% Hispanic. The MDD group (n = 43) had a mean age of 22.7 ± 1.8, mean PROMIS Depression scores of 25.0 ± 6.9, and was 88.4% female, 80.1% white, and 6.7% Hispanic. For later analyses, PROMIS scores were used to partition participants into mild (PROMIS < 23), moderate (PROMIS 23-32), and severe (PROMIS > 32) symptom groups.

2.2. Measures for assessing depression and symptoms in participants

The PROMIS Depression – Short Form (Pilkonis et al., 2011) was created by National Institutes of Health as part of the Roadmap for Medical Research initiative to use item-response-theory methodology to develop psychometrically advanced self-report measures of health outcomes. The adult depression short form consists of an eight-item

Table 1
Demographic Information. This table shows the demographic information in both the healthy control and MDD groups, including sex assigned at birth, gender identity, age, ethnicity, race, SCID score, PROMIS depression and anxiety scores, and prescription of psychoactive drugs.

Characteristic	Specific Descriptor	Healthy Controls	Major Depressive Disorder
Sex Assigned at Birth	Female	34 (72.3%)	38 (88.4%)
	Male	13 (27.7%)	5 (11.6%)
Age (years)	Mean (SD)	22.1 (1.8)	21.9 (2.1)
Ethnicity	Hispanic	3 (6.4%)	6 (14.0%)
	Non-Hispanic	44 (93.3%)	37 (86.0%)
Race	White	38 (80.1%)	33 (76.7%)
	Black	3 (6.4%)	2 (4.7%)
	Asian	5 (10.6%)	3 (7.0%)
	Multiple	1 (2.1%)	3 (7.0%)
	No Response	0 (0%)	2 (4.7%)
PROMIS Depression Score	Mean (SD)	9.3 (1.4)	25.0 (6.9)
Prescribed Psychoactive Drugs	Single	1 (2.1%)	13 (30.0%)
	Multiple	0 (0%)	15 (34.9%)
	No	46 (97.9%)	15 (34.9%)

questionnaire that assesses depressive symptoms over the past seven days. Higher scores indicate greater depressive symptom severity.

The C-SSRS (Posner et al., 2011) and SITBI (Nock et al., 2007) are semi-structured interviews for assessing lifetime history of suicidal thoughts and behaviors and non-suicidal self-injury, respectively. All interviewers received extensive training and supervision from the first author in the administration of this interview and rating of its data. A rigorous protocol developed by the first author was implemented, with an average training period of three to four months before interviewers administered the measure independently. Interviewers conferred with the first author whenever coding questions arose.

The SCID-V (First et al., 2015) is a semi-structured diagnostic interview of current and lifetime DSM-5 psychopathology. For the current study, only the depression module was administered. Research staff were trained by doctoral level clinicians and certified by the first author in the research procedures.

2.3. Extraction and preparation of fecal samples

Upon receipt at Brown University, fecal samples were stored at −80 °C until all samples had been collected. Samples were then thawed, and 300 µL of fecal suspension from each sample was transferred into two plates of the ZymoBIOMICS 96 DNA Kit (Zymo Research) to extract DNA. Samples from the two groups were randomized across the two 96-well plates. Extraction was performed according to manufacturer's protocols, and extracted DNA was measured using the Qubit 3.0 system with Broad-Range DNA reagents (Thermo Fisher Scientific).

2.4. Sequencing

Amplicons of the V4 hypervariable regions of the 16S rRNA gene were generated according to the Earth Microbiome Protocol (Thompson et al., 2017). In brief, 10 µg of extracted DNA from each sample was used as template for triplicate PCR reactions utilizing individually barcoded 515F forward primers (GTGYCAGCMGCCGCGGTAA) with Illumina adapters and the 806R reverse primer (GGACTACNVGGGT-WTCTAAT) with Illumina adapters. Triplicates were combined and measured using the Qubit 3.0 system with Broad-Range DNA Reagents (Thermo Fisher Scientific). Samples were pooled in equimolar concentrations and sent out for 2x250 paired-end sequencing utilizing an Illumina MiSeq system at the University of Rhode Island. We obtained a total of 3,806,054 quality-filtered sequences across all 90 samples. The average sequencing depth was 41,509 reads in the control group and 44,169 in the MDD group. Sequences can be found at the NCBI Short
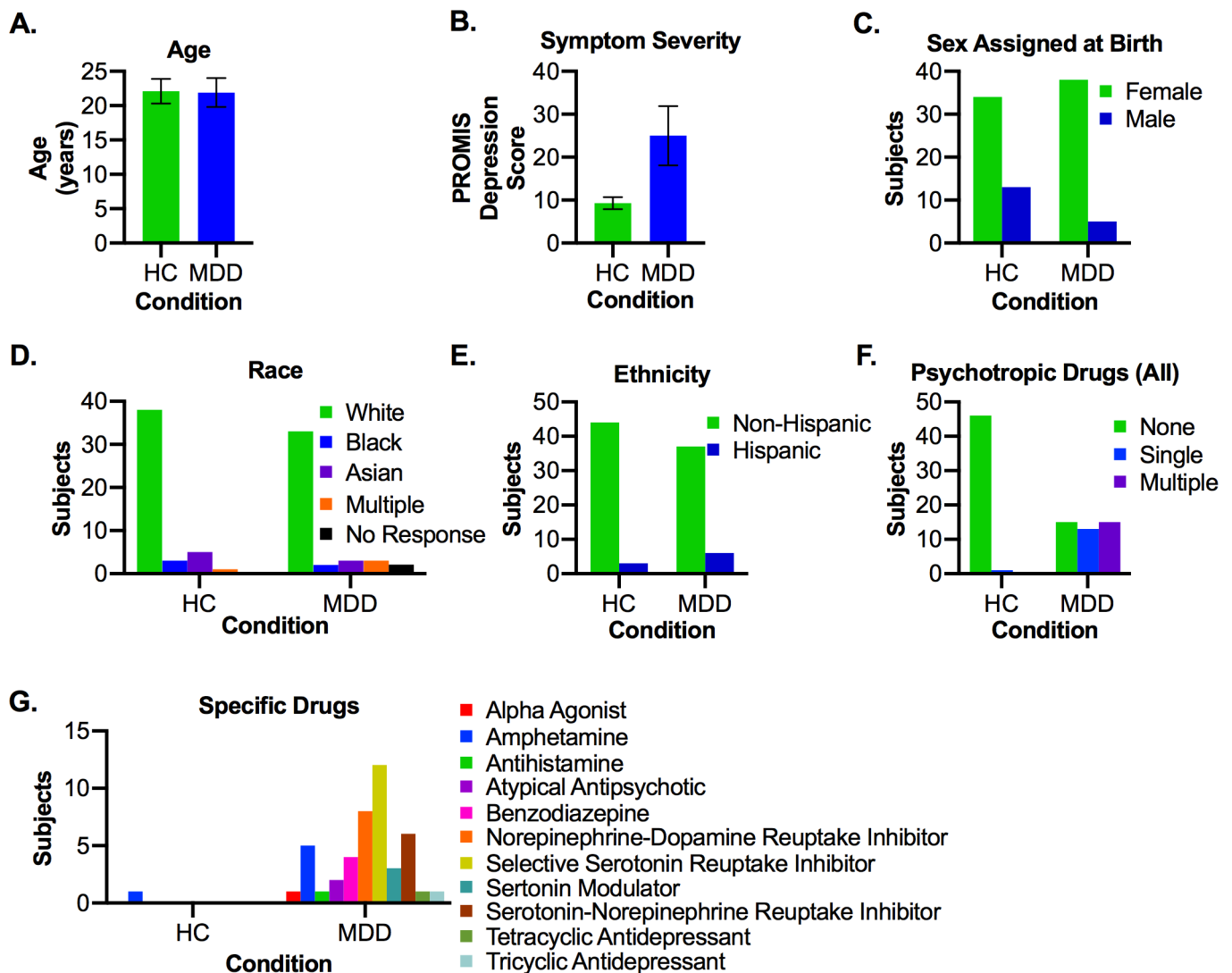
Fig. 1. Demographic characteristics of control and MDD groups. A) Age. B) PROMIS Depression scores. C) Sex assigned at birth. D) Race. E) Ethnicity. F) Number of psychotropic drugs taken. G) Categories of psychotropic drugs taken. *In A-B, bars represent group averages and error bars indicate standard deviation. In G, the sum of all bars in the MDD group may exceed 43, due to subjects taking multiple medications. MDD = Major Depressive Disorder.*

Read Archive under BioProject ID PRJNA591924.

2.5. Microbiome data Analysis

Data was initially processed utilizing the QIIME2 (v2019.7) pipeline (Bolyen et al., 2019). In brief, samples were imported using the *tools* plugin, demultiplexed using the *demux* plugin, and denoised using the *dada2* plugin to obtain amplicon sequence variants (ASVs) (Callahan et al., 2016). Phylogenetic trees were generated using the *phylogeny* plugin and taxonomy was assigned using the *feature-classifier* plugin and the Silva (release 132) 99% identity V4 classifier. Additionally, functional potential was predicted using the *picrust2* plugin (Douglas et al., 1849). The feature table, representative sequences, rooted phylogenetic tree, and taxonomy QIIME2 artifacts were exported, and the feature table, taxonomy, and sample metadata were merged into a unified biom file using the *add-metadata* function of the biom-format package (McDonald et al., 2012).

The exported biom file, phylogenetic tree, and representative sequences were imported into the phyloseq package (v1.28.0) (McMurdie and Holmes, 2013; McMurdie and Holmes, 2012) in R (v3.6.1) using the *import_biom* function. Alpha diversity metrics were calculated using the *estimate_richness* function of phyloseq (Shannon's Diversity Index,

Simpson's Diversity Index, Observed ASVs) and the *estimate_pd* function of the btools package (v0.0.1) (Faith's Phylogenetic Diversity). Beta diversity (Bray Curtis Dissimilarity and both Unweighted and Weighted Unifrac Distances) was calculated using the *phyloseq:distance* function of the vegan package (v2.5–6) (Dixon, 2009), statistically analyzed using the *adonis* function, and subjected to Principal Coordinates Analysis using the *ordinate* function of vegan.

ASV tables were agglomerated at the phylum, class, order, family, and genus levels and exported as relative abundance tables for plotting and analysis (Data S1). The genus-level table was reformatted to conform to the requirements of the Linear Discriminant Analysis Effect Size (LEfSe) web-based tool (Segata et al., 2011), and analysis to identify group biomarkers was performed according to default parameters. Similarly, the MetaCyc pathways output from picrust2 (Data S2) was formatted for and analyzed with LEfSe. All figures were generated using GraphPad Prism v8e, with the exception of Fig. 3B, which is a modified LEfSe output. Statistics were performed in R for beta diversity metrics, LEfSe for differential abundance, and GraphPad Prism for alpha diversity metrics and tests for trends based on psychotropic drug usage or symptom severity. Throughout the text and figures, in cases where discriminant taxa had unclear or nonspecific names at a particular level, the next-higher taxon was included in curly brackets to indicate

its provenance (for example, {*Muribaculaceae*} uncultured bacterium).

2.6. Serum inflammatory cytokines

We utilized the LegendPlex Human Inflammation Panel 1 kit (BioLegend, Lot B291816), which measures levels of IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33, to quantitate serum levels of pro-inflammatory cytokines. Serum was thawed, vortexed, centrifuged, heated at 70 °C for ten minutes, and diluted 1:2 in Assay Buffer before measurement. Serum samples and standards were run in duplicate according to manufacturer's instructions, and data was processed using the LegendPlex analysis software. GraphPad Prism was used to plot data and perform two-tailed t-tests with Welch's correction, and the Bonferroni correction was applied to account for multiple hypothesis testing.

3. Results

3.1. Psychotropic medication usage

We analyzed the microbiomes of 90 participants. Demographics and range of depressive symptom severity based on PROMIS scores of the population can be found in the Materials and Methods and in Fig. 1 and Table 1. In addition to demographics, we also assessed the psychotropic drug usage of the groups. In the MDD group, 15 (34.9%) were not actively taking prescribed psychotropic medications, while 30.0% were taking a single medication, and 34.9% were taking two or more medications (Table S1–2, Fig. 1F). These medications were quite varied, and included a range of both anti-depressants, anxiolytics, and stimulants; in the control group, only one subject was taking a psychotropic medication, specifically an amphetamine for ADHD (Table S2, Fig. 1G).

3.2. Alpha and beta diversity

We started by analyzing the alpha diversity of the control and MDD groups. We first utilized the Observed ASVs metric, which assesses the richness of ASVs found in the samples without considering their phylogenetic relatedness, and Shannon's Diversity Index, which reflects both the richness and evenness of the samples. On both of these measures, there was no significant difference between the two groups (Fig. S1A–B). However, when using Faith's Phylogenetic Diversity, a biodiversity metric which analyzes phylogenetic tree branch length to incorporate relatedness of taxa, we observed a slight but significant decrease in this metric in the MDD group (Fig. 2A). Furthermore, this diversity metric was inversely related to the severity of depressive symptoms (Fig. 2B), and was not impacted by the usage of psychotropic medications (Fig. 2C).

We then utilized metrics of beta diversity to assess whether the healthy and MDD microbiomes were different at a whole-community level. First, we found that there were statistically significant differences in community composition between the two groups based on Bray-Curtis Dissimilarity, which does not consider phylogenetic relatedness of taxa in a sample. Similarly, there was a significant difference in community composition based on Unifrac Distance, which does take relatedness into account. However, the permANOVA indicates that the condition (control or MDD) explains very little of the discrimination between samples ($R^2 = 0.018$ and $R^2 = 0.014$, respectively); accordingly, when plotting these metrics using a Principal Coordinate Analysis (PCoA), we did not observe clear separation of the two groups (Fig. 2D–E). In the case of Bray-Curtis Dissimilarity, there was a clear clustering of several samples in both groups separately from the majority, which could be attributed to the dominance of *Prevotella 9* rather than *Bacteroides* as the predominant genus-level taxon of the phylum Bacteroidetes in those samples (Fig. S1C). This pattern was not observed in the PCoA for Unifrac Distance (Fig. S1D), consistent with the fact that this metric accounts for the two genera's taxonomic

relatedness. Finally, using Weighted Unifrac Distance, which accounts for both phylogenetic relatedness and abundance of taxa, there was no significant difference between the groups and no clear clustering by condition (Fig. S1E).

3.3. Taxonomic composition

We next analyzed the composition of the samples at multiple taxonomic levels to assess whether there were differences in the communities of depressed and healthy subjects. We utilized the Linear Discriminant Analysis Effect Size (LEfSe) tool to identify taxa that were biomarkers of each group and found a range of discriminating taxa across all taxonomic levels (Fig. 3, Table S4). At the phylum level, controls were enriched in Firmicutes and the MDD subjects were enriched in Bacteroidetes (Fig. 4A–C, Table S4). Similar findings were obtained at the class and order levels (Fig. S2A–B), with the class Clostridia and order Clostridiales of Firmicutes associated with controls while the class Bacteroidia and order Bacteroidales of Bacteroidetes were associated with depressed subjects (Fig. S2C–F, Table S4). Additionally, within the phylum Proteobacteria, the order Rhodospirillales of the class Alphaproteobacteria was associated with controls, while the class Gammaproteobacteria was associated with the MDD group (Fig. S2G–H). At the family level (Fig. S2I, Table S4), the Clostridiales families *Ruminococcaceae* and *Christensenellaceae* were associated with the control group (Fig. S2J–K), as well as the Bacteroidetes family *Barnesiellaceae* and an uncultured family of the order Rhodospirillales (Fig. S2L–M), while the family *Enterococcaceae* (of the Bacilli-Lactobacillales lineage of Firmicutes) was associated with the MDD group (Fig. S2N).

A number of trends at the family level were also represented in their subordinate genera, although there were some contrasting patterns as well (Fig. 5A, Table S4). First, the four most abundant genera within the Firmicutes family *Ruminococcaceae* (*Faecalibacterium*, *Subdoligranulum*, *[Eubacterium] coprostanoligenes* group, and *Ruminococcus 1*) were associated with healthy controls (Fig. 5B–E), as well as the less-abundant genus-level taxon CAG-352 (Fig. 5F). On the contrary, only a single *Ruminococcaceae* genus, *Flavonifractor*, was associated with MDD subjects (Fig. 5P). Additionally, while the related Clostridiales family *Lachnospiraceae* itself was not associated with either group, a number of its member genera were associated with healthy controls, including *Fusicatenibacter*, *Tyzzerella 3*, and *[Eubacterium] ventriosum* group (Fig. 5G–I). In contrast, the *Lachnospiraceae* genus *Sellimonas* was associated with the MDD group (Fig. 5Q). The genus-level taxon {*Lachnospiraceae*} UCG-001 was also called as associated with the MDD group, but a closer examination reveals that this taxon is in fact more highly present and abundant in the control group and the misidentification is likely due to one extreme outlier in the MDD group (Fig. 5O).

Other discriminatory genus-level taxa within the phylum Firmicutes included an uncultured organism of the {Clostridiales} vadin BB60 family and the R-7 group of *Christensenellaceae*, both associated with controls (Fig. 5J–K), and *Enterococcus*, which was associated with MDD subjects (Fig. 5R). Within the phylum Bacteroidetes, *Barnesiella* and an uncultured bacterium of the *Muribaculaceae* family were associated with controls (Fig. 5L–M), while no genus-level taxa were associated with MDD subjects. Finally, within Proteobacteria, the Deltaproteobacteria genus *Desulfovibrio* was associated with healthy controls (Fig. 5N). As considered in greater depth in the discussion, these changes generally appear to reflect a loss of protective bacteria and an increase in pro-inflammatory bacteria in the MDD group.

3.4. Impact of psychotropic medication

We then examined whether any of the associations of taxa with depressed subjects were driven by the consumption of psychotropic medication. We compared the taxa that were identified as significant between the healthy controls, depressed subjects taking no medication,
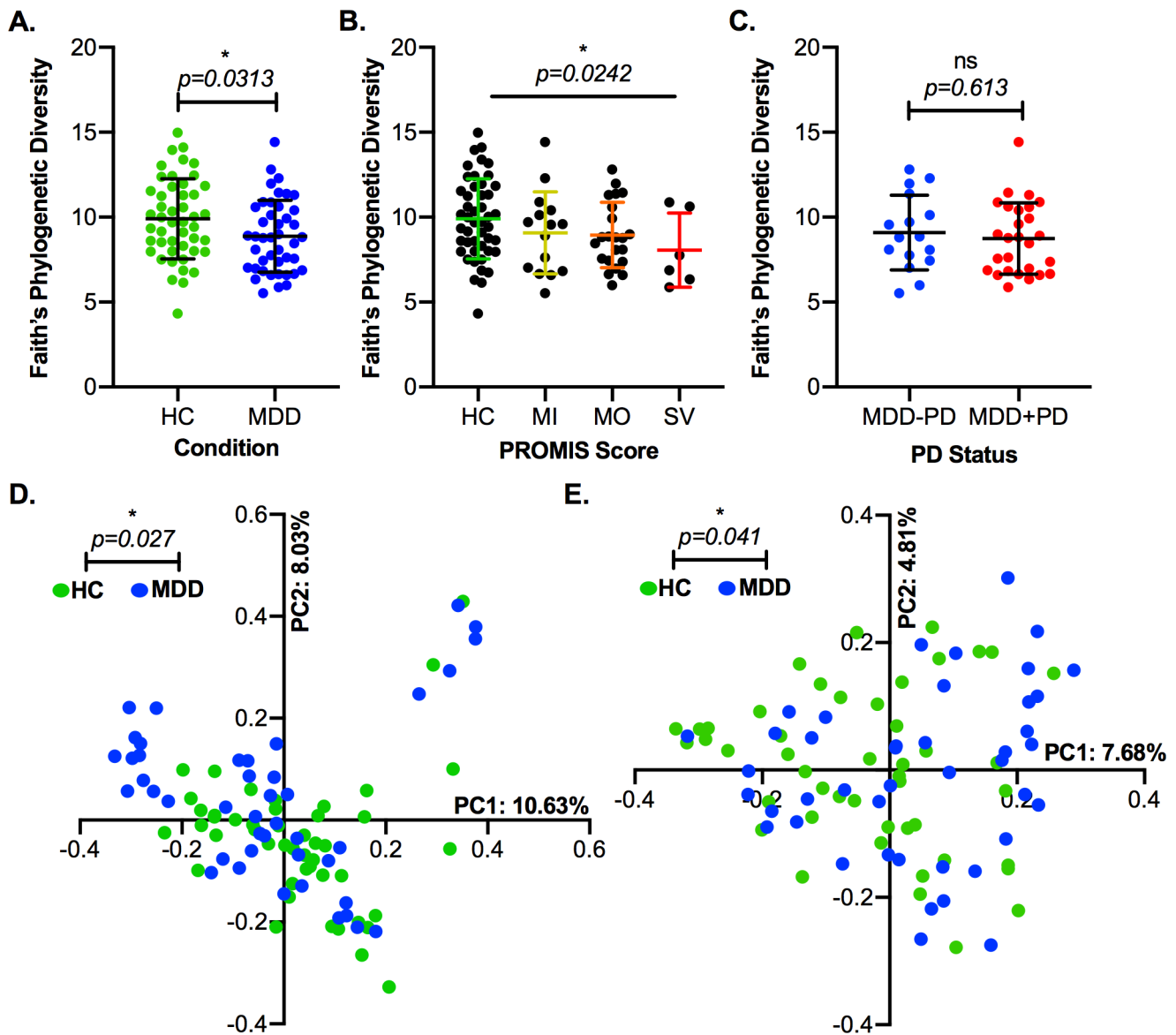
Fig. 2. Phylogenetic Diversity is Reduced in Subjects with MDD. A) Faith's Phylogenetic Diversity in control and MDD groups (*, $p = 0.0313$, t-test with Welch's correction). B) Faith's Phylogenetic Diversity in healthy controls and depressed subjects with mild, moderate, and severe symptoms according to PROMIS depression scores (*, $p = 0.0242$, ANOVA post-test for linear trend of column means). C) Faith's Phylogenetic Diversity in depressed subjects taking no psychotropic medication and depressed subjects taking one or more psychotropic drugs (ns, $p = 0.613$, t-test with Welch's correction). D) Principal Coordinates 1 and 2 of Bray-Curtis Dissimilarity, with points colored according to their source sample's condition group (*, $p = 0.027$, $R^2 = 0.01$, permANOVA). E) Principal Coordinates 1 and 2 of Unweighted Unifrac Distance, with points colored according to their source sample's condition group (*, $p = 0.041$, $R^2 = 0.0106$, permANOVA). In A-C, the central line indicates the group average and error bars indicate standard deviation. Abbreviations: HC = Healthy Control, MDD = Major Depressive Disorder, MI = Mild, MO = Moderate, SV = Severe, PD = Psychotropic Drug, PC = Principal Coordinate. Faith's Phylogenetic Diversity is a metric of biodiversity that incorporates phylogenetic relationships. Principal Coordinates Analysis is a method of multidimensional scaling that attempts to find the main axes through a complex matrix, in this case of beta diversity values. Bray-Curtis Dissimilarity compares communities without incorporating phylogenetic relationships between taxa. Unweighted Unifrac Distance compares communities with the inclusion of phylogenetic data but does not weight by abundance.

and depressed subjects taking one or more medications. Unfortunately, as the number and types of medications varied significantly, we could not assess the impacts of specific classes or combinations of medications.

Generally, we found that the trends observed in the depressed subjects were present in both the medicated and unmedicated groups, although the effect was sometimes stronger in one group than the other (Fig. S3). Specifically, the MDD-associated reductions observed in the phylum Firmicutes, the class Clostridia, the order Clostridiales, the family *Ruminococcaceae*, and the genus-level taxa *Faecalibacterium*, and *[Eubacterium] coprostanoligenes* group, were slightly stronger in the medicated group (Fig. S3A–F). Similarly, the depression-associated

increases in the phylum Bacteroidetes, the class Bacteroidia, and the order Bacteroidales, were somewhat stronger in this group (Fig. S3G–I). In particular, the changes in the genera *Flavonifractor* and *Sellimonas* appeared to be driven primarily by the medicated group (Fig. S3J–K). On the other hand, the MDD-associated reductions in the families *Christensenellaceae* and *Barnesiellaceae*, and genera *Christensenellaceae* R-7 group, *Barnesiella*, and an uncultured organism of the Clostridiales vadin BB60 family-level taxon appear somewhat stronger in the unmedicated group (Fig. S3L–P). In the case of the genus *Fusicatenibacter*, reductions in the depressed group appeared to be primarily driven by the unmedicated subjects (Fig.S3Q). There were also a number of cases in which there were no apparent differences between the depressed
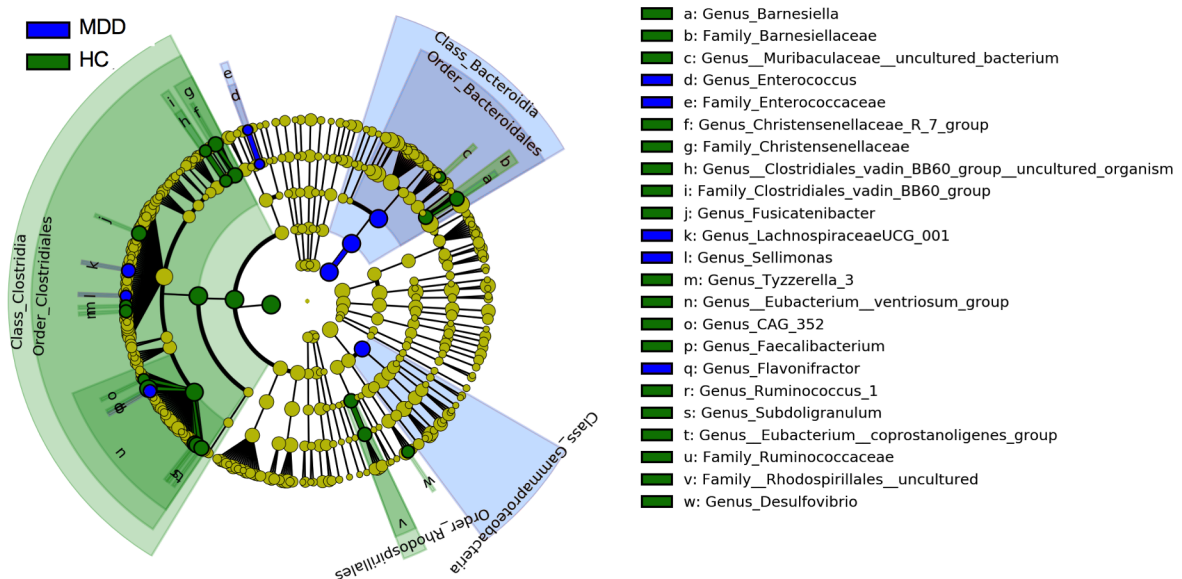
A.
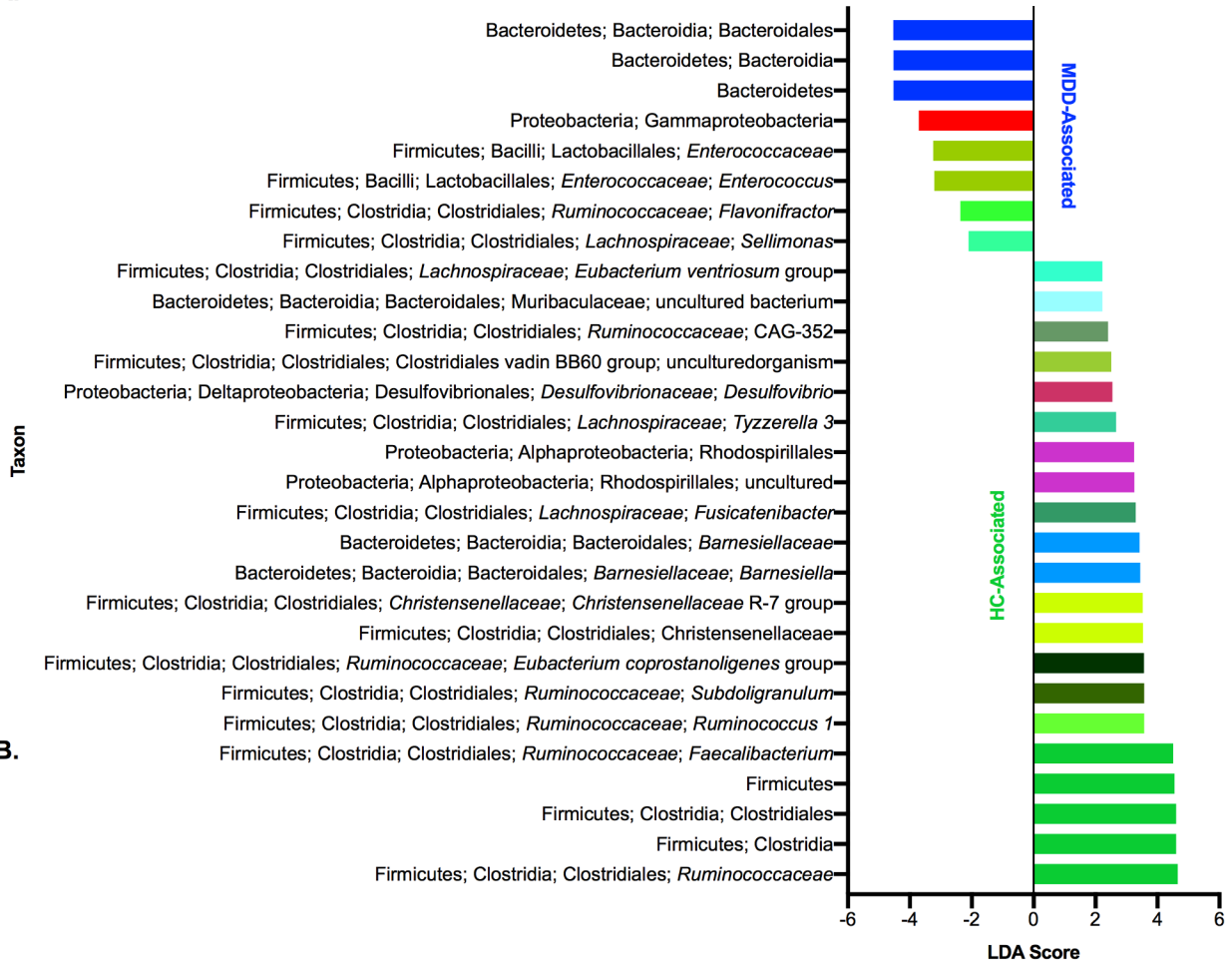
B.

Fig. 3. Differential Taxa are Associated with Control and MDD Subjects. A) Taxa identified by LEfSe as biomarkers of samples from the control or MDD groups (*cutoffs were LDA ≥ 2 and p-value ≤ 0.05*). B) Cladogram indicating the phylogenetic relatedness of the discriminant taxa. *Colors in A) correspond to the taxonomic position, matching the coloring used in Figures 4, 5, and S2. Abbreviations: LEfSe: Linear Discriminant Analysis Effect Size, MDD = Major Depressive Disorder. LEfSe is a biomarker discovery tool used to identify features (taxa, in this case) from high-dimensional genomic data that characterize differences between two or more biological conditions.*
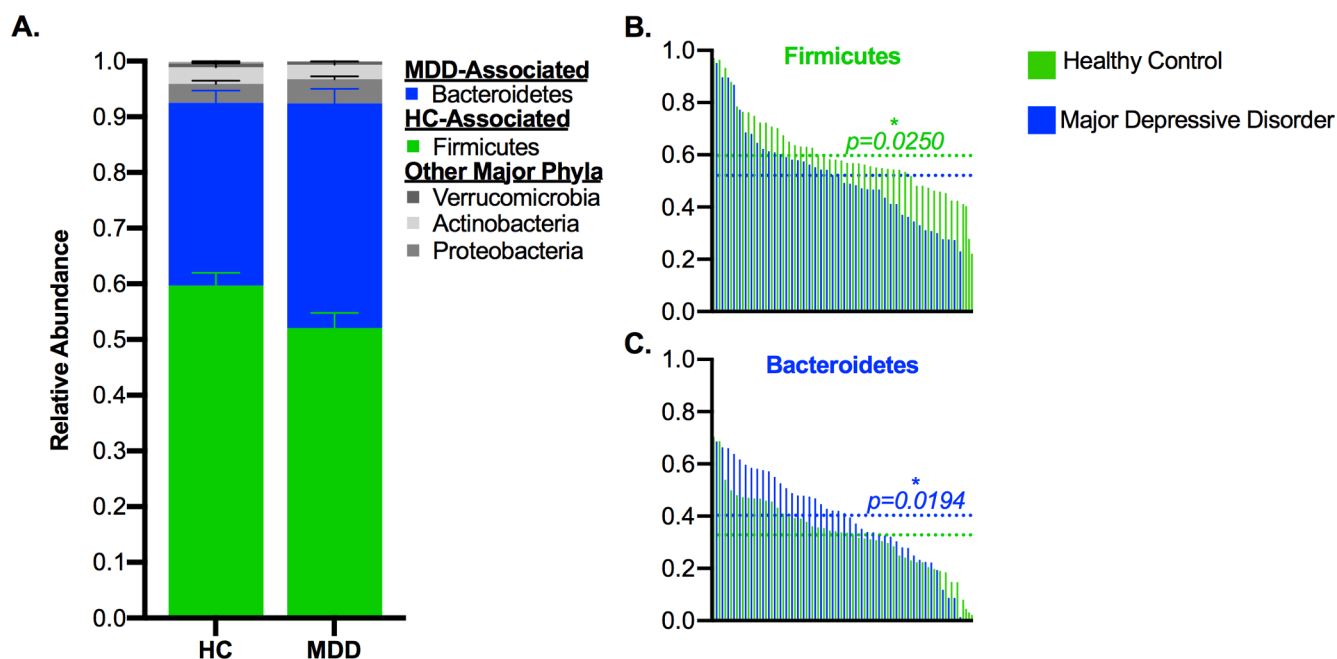
Fig. 4. Major Phyla Are Differentially Abundant Between Healthy and MDD Subjects. A) Stacked bar plot indicating the average relative abundance of phyla within the control and MDD groups, with discriminant phyla identified by LEfSe highlighted in color. B-C) Phyla that were discriminant of the groups, with control and MDD samples interleaved by ranked abundance of each taxon and dotted lines indicating the average relative abundance by group. *In A, error bars indicate standard error of the mean. In B-C, the text color indicates the group that the phylum was associated with, and p-values are from LEfSe. Abbreviations: MDD = Major Depressive Disorder, LEfSe = Linear Discriminant Analysis Effect Size, HC = Healthy Control. LEfSe is a biomarker discovery tool used to identify features (taxa, in this case) from high-dimensional genomic data that characterize differences between two or more biological conditions.*

subjects based on medication (Fig. S3R–AA). Finally, in the case of the family *Enterococcaceae* and its daughter genus *Enterococcus*, levels of these taxa were found only in medicated subjects, suggesting that this group may drive the effect (Fig. S3AB–AC); however, as they were detected in so few samples, it is difficult to make this judgment.

Overall, the differences between depressed and healthy subjects could not be specifically attributed to psychotropic medications, as changes were typically present in both medicated and unmedicated subjects, although they may play a role in some cases. Additionally, we were unable to examine the impacts of specific classes of psychotropic drugs, given the variety and combinations of medications taken by participants.

3.5. Impact of depression severity

While psychotropic medication use may have had some impact on taxonomic trends, we also found that depression severity could significantly confound this interpretation. Based on PROMIS Depression scores, the proportion of subjects taking psychotropic medication increased based on their symptom severity: 46.7% of the 15 subjects with mild symptoms were taking psychotropic medication, compared with 68.2% of the 22 subjects with moderate symptoms and 83.3% of the 6 subjects with severe symptoms (Fig. S4A). Accordingly, the proportions of subjects with mild symptoms was higher in the unmedicated group (53.3% vs. 28.6%), while the proportion with severe symptoms was lower (6.7% vs. 17.9%) (Fig. S4B). Therefore, we decided to assess whether any of the observed trends tracked with symptom severity, which might explain differences better than whether or not depressed subjects were taking one or more of a wide range of psychotropic drugs with different mechanisms of action.

In fact, despite interindividual variation, the observed changes were more exaggerated in subjects with higher depressive symptom severity scores in the majority of taxa (Fig. 6A, S5A); this trend was significant in most of these cases, although a few fell just short of statistical significance (Table S3). Specifically, the phylum Firmicutes, class

Clostridia, order Clostridiales, family *Ruminococcaceae* and its member genera *Faecalibacterium*, *[Eubacterium] coprostanoligenes* group, *Subdoligranulum*, and *Ruminococcus 1*, and family *Christensenellaceae* and member genus-level taxon *Christensenellaceae* R-7 group were more reduced in subjects with more severe symptoms (Fig. 6B–G, S5B–K). In the case of *Fusicatenibacter*, reductions were most pronounced in the subjects with severe symptoms, although there was not a consistent trend in the mild and moderate symptom groups (Fig. S5L). In the other direction, the phylum Bacteroidetes, classes Bacteroidia and Gammaproteobacteria, order Bacteroidales, and genera *Flavonifractor* and *Sellimonas* were more abundant in subjects with more severe symptoms (Fig. 6H–L, S5M–R). There were also a few cases in which such a trend was not present or where there were very few depressed samples with detectable levels of a given taxon, making it difficult to assess whether their abundance aligns with symptom severity. (Fig. S5S–AD).

In general, these results suggest that symptom severity tracks with changes in a number of discriminatory taxa. While the impact of psychotropic drug usage on this pattern cannot be ruled out due to the higher levels of medication utilization in subjects with more severe depressive symptoms (Fig. S4A–B), the high degree of variability in the number and classes of drugs used (Fig. 1, Table S2–3) tends to suggest that the effect is more likely related to symptom severity.

3.6. Functional predictions

We utilized Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) to predict the functional potential of the healthy and depressed microbial communities based on the 16S rRNA gene content. It should be noted that this is only a prediction based on the inference from the 16S rRNA content and cannot definitively measure functional potential or transcriptional activity. When assessing function at the MetaCyc pathway level, LEfSe detected a number of pathways that were associated with each group. Pathways associated with the MDD subjects tended to be related to vitamin (folate and thiamine) biosynthesis, LPS biosynthesis, and long-
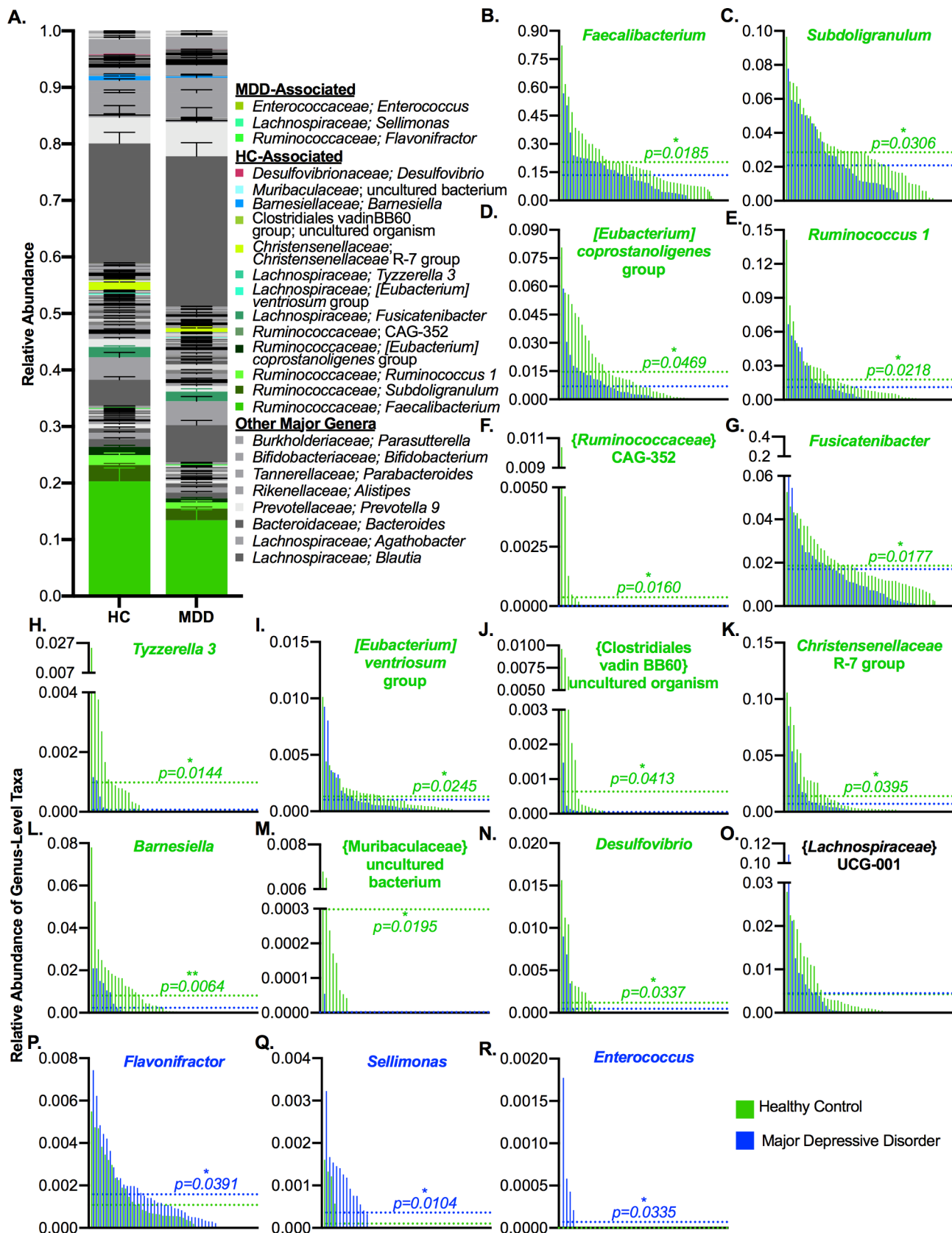
Fig. 5. Numerous Genera Are Differentially Abundant Between Healthy and MDD Subjects. A) Stacked bar plot indicating the average relative abundance of genera within the control and MDD groups, with discriminant phyla identified by LEfSe highlighted in color. B-R) Genera that were discriminant of the groups, with control and MDD samples interleaved by ranked abundance of each taxon and dotted lines indicating the average relative abundance by group. *In A, stacking is done by phylogeny, so all genera are grouped by their higher taxonomic ranks. Error bars indicate standard error of the mean. In B-R, the text color indicates the group that the genus was associated with, and p-values are from LEfSe. Abbreviations: MDD = Major Depressive Disorder, LEfSe = Linear Discriminant Analysis Effect Size, HC = Healthy Control. LEfSe is a biomarker discovery tool used to identify features (taxa, in this case) from high-dimensional genomic data that characterize differences between two or more biological conditions.*
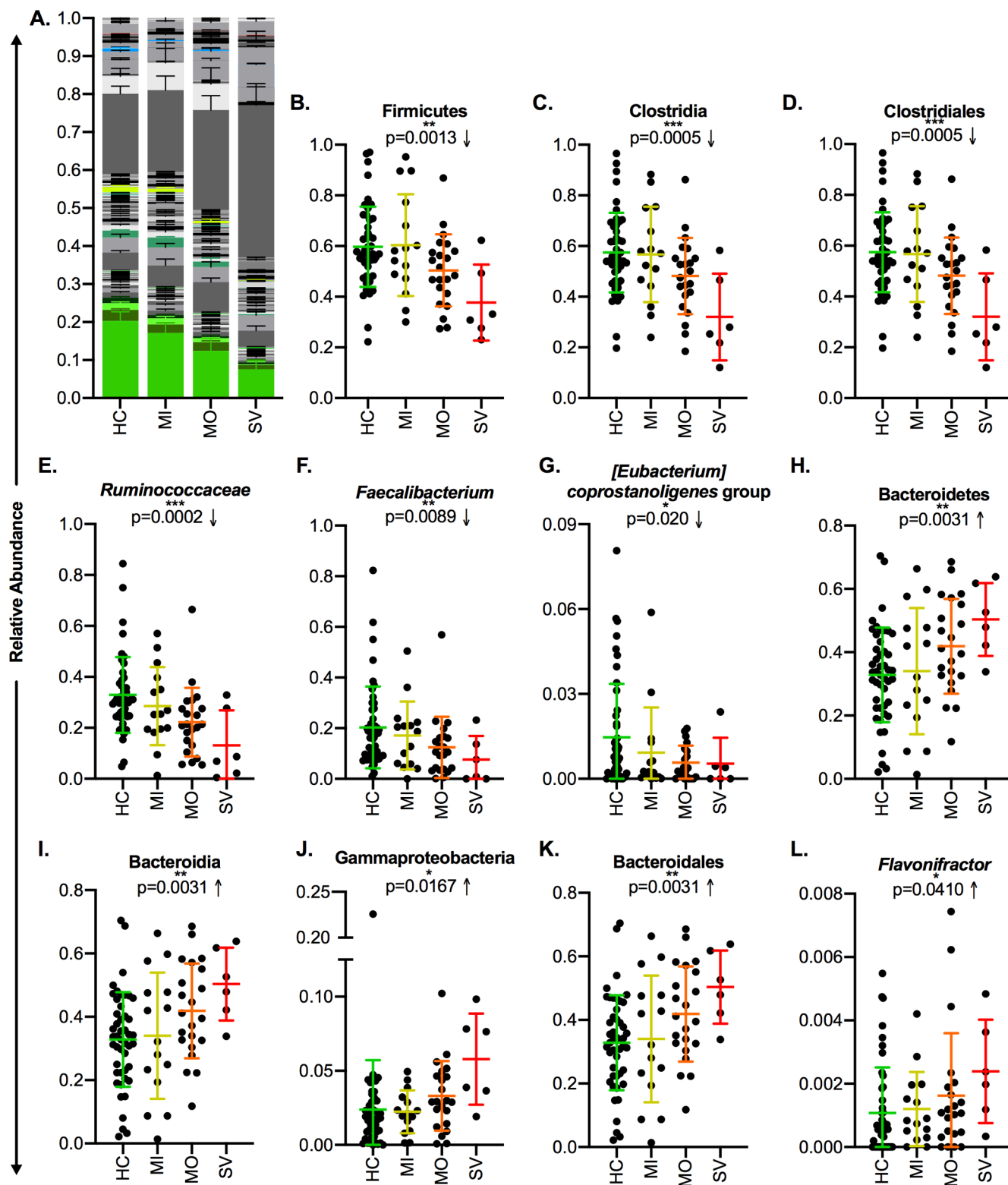
Fig. 6. Taxonomic Differences in MDD Subjects Track Significantly with Symptom Severity. A) Stacked bar plot indicating the average relative abundance of genera within the control subjects and MDD subjects with mild, moderate, or severe symptoms according to PROMIS depression scores, with genera that discriminated the control and MDD groups identified by LEfSe highlighted in color. B-L) Relative abundances of discriminant taxa in the control, MDD-mild symptoms, MDD-moderate symptoms, and MDD-severe symptoms groups. *In A, stacking is done by phylogeny, so all genera are grouped by their higher taxonomic ranks. Error bars indicate standard error of the mean. In B-L, central lines indicate the group mean and error bars indicate standard deviation, p-values are from the ANOVA post-test for linear trend of column means, and arrows indicate the direction of the trend. Abbreviations: MDD = Major Depressive Disorder, LEfSe = Linear Discriminant Analysis Effect Size, HC = Healthy Control, MI = Mild, MO = Moderate, SV = Severe. LEfSe is a biomarker discovery tool used to identify features (taxa, in this case) from high-dimensional genomic data that characterize differences between two or more biological conditions.*

chain fatty acid biosynthesis. On the other hand, pathways associated with the healthy subjects tended to be related to fermentation to short chain fatty acids, phospholipid biosynthesis, nucleic acid metabolism, and aliphatic amino acid biosynthesis (Fig. S6). Of particular note is the association with healthy controls of PWY 5676, "acetyl CoA fermentation to butanoate II", as fermentation of acetyl-CoA is the dominant pathway by which the gut microbiota, including *Faecalibacterium prausnitzii*, produce the anti-inflammatory short-chain fatty acid butyrate (Vital et al., 2014; Anand et al., 2016). The control group was also enriched in the PWY5100, "pyruvate fermentation to acetate and lactate II", which produces another major microbial SCFA, acetate. Additionally, the MDD group's association with LPS production (PWY1269 – "CMP-3-deoxy-D-*manno*-octulosonate biosynthesis", NAGLIPASYN – "lipid IVA biosynthesis [*E. coli*]", PWY6467 – "KDO transfer to lipid IVA III [*Chlamydia*]", PWY7323 – "superpathway of GDP-mannose-derived O-antigen building blocks"), likely due to the enrichment in the Gram-negative phylum Bacteroidetes and class Gammaproteobacteria, is notable. In particular, Proteobacteria-derived LPS is known to be immunogenic and has been linked to chronic inflammation (d'Hennezel et al., 2017; Vatanen et al., 2016).

3.7. Circulating pro-inflammatory cytokines

We utilized a commercially-available kit to measure a range of important inflammation-related cytokines (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33) present in serum collected from the participants. We did not observe any significant differences in any of the measured cytokines between the MDD and control groups (Fig. S7).

4. Discussion

Depression has been previously found to be associated with differences in the gut microbiota, and our study adds to this body of work. However, a key concept in microbiome research is the heterogeneity of microbial populations between various groups of people; therefore, as we compare our conclusions to previous studies, it is important to consider the factors that make this study unique relative to other work. First, the majority of previous MDD-microbiome studies have been undertaken in Chinese populations, with a few others studying European subjects, while our study focused on an American population (Kelly et al., 2016; Jiang et al., 2015; Huang et al., 2018; Zheng et al., 2016; Naseribafrouei et al., 2014; Chen et al., 2018; Valles-Colomer et al., 2019; Lai et al., 2019; Rong et al., 2019; Lin et al., 2017). Given that the underlying microbiome can differ significantly by geography (Costello et al., 2009; Gupta et al., 2017; Yatsunenko et al., 2012), it is important to study the impacts of disease in a range of populations. Second, most previous studies have studied a wider and older age range than our own, which recruited only subjects between the ages of 18 and 25. As the microbiome can change through the lifespan (Costello et al., 2009; Yatsunenko et al., 2012; Odamaki et al., 2016), recruitment of a narrow age range can limit underlying noise and increase power to detect differences between groups. Third, we were able to recruit a notable subset of MDD participants who were not taking psychotropic medication, which allowed us to compare this group to the larger group of participants who were using these drugs, which most previous studies were often unable to do. Finally, we were able to recruit MDD participants with a range of depressive symptom severities, which allowed us to assess whether the changes we observed in the MDD group were related to this metric. Based on this design, we observed a number of notable differences between MDD and control participants, and further found that many of these changes may track with symptom severity.

Despite demographic differences, many of our results do align with observations made previously in other populations. Most notably, we found that *Faecalibacterium* levels were reduced in subjects with MDD,

supporting previous work linking lower levels of this genus to depression or bipolar disorder and lower quality of life (Evans et al., 2017; Chen et al., 2018; Valles-Colomer et al., 2019; Jiang et al., 2015; Huang et al., 2018; Zheng et al., 2016). We also found this pattern in a number of related genera within the family *Ruminococcaceae*, including *Subdoligranulum*, *Ruminococcus 1*, and *[Eubacterium] coprostanoligenes* group. There was a member of this family – *Flavonifractor* – which was instead more abundant in the MDD group, supporting previous work finding that higher levels of this genus are associated with depression, bipolar disorder, and lower quality of life (Coello et al., 2019; Jiang et al., 2015; Valles-Colomer et al., 2019). Remarkably, we found that many of differences that we observed were exacerbated in subjects with more severe depressive symptoms, which had previously only been observed for *Faecalibacterium* in major depressive disorder and bipolar disorder (Evans et al., 2017; Jiang et al., 2015). In general, our results, particularly the negative correlation between *Faecalibacterium* levels and depressive symptoms and the association of MDD with increased levels of *Flavonifractor* align most closely with those of Jiang et al. (2015). This may relate to the fact that this study utilized a subject population closer in age to our own, with average age in the mid-twenties, compared with other studies where the average ages ranged from mid-thirties to late forties; notably, across geographies and ethnic groups, microbiome composition tends to shift to an older phenotype by the age of forty (Yadav et al., 2016). Further work to examine whether microbiome alterations in depressed subjects are related to age may be warranted.

There were also a few trends that were contradictory to prior data. In particular, while several studies have found lower levels of the phylum Bacteroidetes and higher levels of the phylum Firmicutes in depressed subjects (Zheng et al., 2016; Naseribafrouei et al., 2014; Chen et al., 2018; Lai et al., 2019; Rong et al., 2019; Lin et al., 2017), we found the opposite trend. Furthermore, while Jiang et al. did find that Bacteroidetes were higher in depressed subjects, this was in fact driven by increases in the families *Rikenellaceae* and *Porphyromonadaceae*, while *Bacteroidaceae* was actually slightly reduced (Jiang et al., 2015); this contrasts with our data, in which increases in the levels of Bacteroidetes in our MDD group were largely driven by increases in *Bacteroidaceae* and *Prevotellaceae*, although these differences were not significant at the family level. Additionally, while some studies have found increases in the Gammaproteobacteria family of *Enterobacteriaceae* in depressed subjects (Jiang et al., 2015), we did not observe such a change. While we found that Gammaproteobacteria was associated with the MDD group, an examination of the data suggests that this increase was driven by the genus *Parasutterella* of the family *Burkholderiaceae*. Finally, while lower levels of *Fusicatenibacter* were found in a previous study to be associated with lower quality-of-life scores and potentially with depression, the relationship with depression was not found in the non-medicated subset of subjects (Valles-Colomer et al., 2019). This is in contrast to our results, where we instead found that low levels of this genus were primarily found in the non-medicated MDD subjects. Importantly, as noted previously, our study is fairly demographically distinct from previous studies of the gut microbiota in depression, which could be responsible for some of the disparities between our results and those of prior studies. In particular, geographic location significantly impacts lifestyle factors, including diet, that can substantially change the underlying microbial composition of a population. For example, previous work has established that the microbiomes of so-called western populations are distinct from those of non-western groups, and furthermore that the gut microbiota of non-western immigrants converges with those of native westerners over time and generations due to lifestyle and dietary changes (Yadav et al., 2016; Nam et al., 2011; Vangay et al., 2018).

In light of the links between MDD and chronic inflammation, a number of microbiota differences observed in our study are notable. Perhaps most interesting is the relationship between MDD subjects and lower levels of the family *Ruminococcaceae* and its daughter genera

Faecalibacterium, *Subdoligranulum*, *Ruminococcus 1*, and *[Eubacterium] coprostanoligenes* group. The genus *Faecalibacterium* includes only one named species, *F. prausnitzii*, which has been demonstrated to have anti-inflammatory properties (Qiu et al., 2013; Quevrain et al., 2016; Sokol et al., 2008; Zhou et al., 2018). Importantly, it produces the short-chain fatty acid butyrate (Louis and Flint, 2017), which serves as a colonic fuel source, fosters immunoregulation, and promotes epithelial barrier integrity (Arpaia et al., 2013; Furusawa et al., 2013; Hamer et al., 2008; Leonel and Alvarez-Leite, 2012; Parada Venegas et al., 2019; VanHook, 2015; Wang et al., 2012; Zhang et al., 2016). In fact, lower levels of this genus and species have been associated with IBD, *Clostridiodes difficile* colitis, autoimmune disorders, and atherosclerotic cardiovascular disease (Kostic et al., 2014; Brown et al., 2011; de Paiva et al., 2016; Jie et al., 2017; Sokol et al., 2008; Gevers et al., 2014; Kabeerdoss et al., 2013; Quince et al., 2015; Takahashi et al., 2016; Kang et al., 2010), in addition to mental health disorders including depression (Jiang et al., 2018; Evans et al., 2017; Jiang et al., 2015; Huang et al., 2018; Zheng et al., 2016). Similarly, *Subdoligranulum* includes only a single named species, *S. variable*, which is also known to produce butyrate (Louis and Flint, 2017) and has been negatively associated with IBD (Brown et al., 2011; Quince et al., 2015,; Papa et al., 2012). Additionally, it has previously been found to correlate negatively with depressive symptoms (Chen et al., 2019). The genus-level taxon *Ruminococcus 1* includes the species *R. albus* and *R. callidus*, both of which have also been negatively linked to IBD (Kang et al., 2010), although they do not themselves produce butyrate (Louis and Flint, 2017).

The relationship between *[Eubacterium] coprostanoligenes* group and MDD is less clear, as this genus-level taxon has not been specifically linked to depression or inflammation. However, like *Subdoligranulum*, its abundance has been negatively correlated with depressive symptoms (Chen et al., 2019). It is named for primary component species *E. coprostanoligenes*, which is known for its ability to reduce cholesterol to coprostanol, which is less-well-absorbed by the host (Freier et al., 1994; Ren et al., 1996). Fecal coprostanol levels or the ratio of fecal coprostanol/coprostanone to cholesterol have been found to be reduced in Crohn's disease, ulcerative colitis, and *Clostridioides difficile* colitis (Sundqvist et al., 2019; Reddy et al., 1977; Santoru et al., 2017; Antharam et al., 2016; Midtvedt et al., 2013), suggesting that a reduction in *E. coprostanoligenes* or related species could contribute to inflammation through increased colonic cholesterol levels. Evidence suggests that this may relate to serum cholesterol as well, as administration of *E. coprostanoligenes* to mice and rabbits decreased serum cholesterol levels (Li et al., 1998; Li et al., 1995) and a human study found that serum cholesterol was inversely related to fecal coprostanol:cholesterol ratios (Sekimoto et al., 1983). However, while higher levels of cholesterol have generally been thought to be negative for health, there is some evidence that very low cholesterol levels are associated with severe depression, although the evidence is mixed and may depend on gender and the type of cholesterol (HDL vs LDL) (Deisenhammer et al., 2004; Kim and Myint, 2004; Sansone, 2008).

Flavonifractor, the sole member of *Ruminococcaceae* that was associated with the depressed subjects, has previously been linked to lower quality of life scores and MDD (Jiang et al., 2015; Valles-Colomer et al., 2019) as well as bipolar disorder (Coello et al., 2019) and generalized anxiety disorder (Jiang et al., 2018). This genus, which currently includes the single named species *F. plautii* (formerly *Clostridium orbiscindens* and *Eubacterium plautii*) (Carlier et al., 2010), has also previously been linked to various autoimmune disorders (He et al., 2016; Huang et al., 2019; Sun et al., 2019; Mancabelli et al., 2017), chronic kidney disease (Lun et al., 2019), and colorectal cancer (Ai et al., 2019; Gupta et al.,). Furthermore, *F. plautii* was demonstrated *in vitro* to have epithelial invasive potential (Armstrong et al., 2019). Therefore, there is significant evidence to suggest that unlike its generally anti-inflammatory relatives, *Flavonifractor* may be associated with disease despite its ability to produce butyrate under some conditions (Carlier et al., 2010; Klaring et al., 2013). As suggested in other work (Coello

et al., 2019; Gupta et al.,), this is possibly related to the genus' eponymous capacity for cleaving flavonoids that reach the colon, including antioxidants such as quercetin, although disentangling the impacts of flavonoids and their microbial breakdown products on inflammation *in vivo* is difficult (Carlier et al., 2010; Winter et al., 1991; Schoefer et al., 2003; Tunon et al., 2009; Loke et al., 2008; Peng et al., 2014; Jaganath et al., 2009).

In addition to the *Ruminococcaceae*, some other members of Firmicutes were altered in the depressed group, including a few members of the family *Lachnospiraceae*. The genus-level taxon *Tyzzerella 3* was associated with the control subjects, although it has previously been linked to generalized anxiety disorder (Chen et al., 2019); however, it was also linked to healthy controls in a study of chronic kidney disease (Lun et al., 2019). *[Eubacterium] ventriosum* group was also associated with controls, and this genus-level taxon has been found to produce butyrate and inversely correlate with the inflammatory cytokines IL-6 and IL-8(Barcenilla et al., 2000; Biagi et al., 2010). Finally, *Fusicatenibacter* was also more abundant in the control group, and was particularly reduced in the subjects with severe symptoms; its primary member species *F. saccharivorans* has previously been found to be reduced in subjects with active IBD and colorectal cancer (Takeshita et al., 2016; Zhang et al., 2018). On the other hand, the genus *Sellimonas* was associated with the MDD subjects, and has been previously linked to rheumatoid arthritis (Sun et al., 2019) and chronic kidney disease (Lun et al., 2019).

Previous work has demonstrated that the genera *Barnesiella* and *Christensenellaceae R-7* were associated with healthy controls in comparison to various inflammation-related gut diseases, including IBD, colorectal cancer, and *C. difficile* colitis (Mancabelli et al., 2017); however, these taxa have not previously been linked to depression. Finally, in the family Proteobacteria, the genus *Desulfovibrio* was associated with the healthy control subjects, although in previous studies it has been found to be associated with IBD and experimental colitis models (Rowan et al., 2010; Figliuolo et al., 2017; Millien et al., 2019); however, in a study of generalized anxiety disorder, its source family of *Desulfovibrionaceae* was associated with healthy controls (Jiang et al., 2018). Additionally, the class Gammaproteobacteria was associated with MDD, which was largely driven by increases in the family *Burkholderiaceae* and genus *Parasutterella*, which has itself been linked to MDD (Jiang et al., 2015). Relatedly, we also observed a predicted enrichment in LPS biosynthesis pathways in the MDD group, which was also observed by Huang et al. (2018); the LPS of members of Gammaproteobacteria is known to be immunogenic relative to that of some commensal Gram-negative bacteria such as *Bacteroides* species (d'Hennezel et al., 2017; Vatanen et al., 2016), and depressed subjects have been found to have increased serum immunoglobulin A and M responses against the LPS of members of this class (Maes et al., 2008; Maes et al., 2012).

Some limitations of this study must be acknowledged. First, while there are clear trends that significantly discriminatory taxa became increasingly divergent with increasing symptom severity, we cannot completely disentangle this phenomenon from the taking of psychotropic drugs given the strong degree of overlap between symptom severity and medication usage. Future work specifically focusing on newly-diagnosed subjects who have not previously taken psychotropic drugs would be beneficial in understanding the relationship between the microbiota, depressive symptom severity, and medication. Additionally, our study was not designed to study potential microbiome differences between subjects who respond to psychotropic treatment and non-responders – in fact, given the overlap between symptom severity and psychotropic treatment, our study potentially included many non-responders – but this would also be an important element in understanding the interplay between these factors.

Additionally, we utilized 16S rRNA sequencing, which can generally only identify taxa down to the genus level, so we may be missing important species- or strain-level differences between the communities.

Furthermore, while we can use taxonomic composition to predict the gene content of the communities, full metagenomics and transcriptomics would be required to comment further on changes in the functional potential or transcriptional activity, such as potentially reduced capacity for SCFA production in depressed subjects. Moreover, while we did assay circulating cytokine levels as a potential marker of systemic inflammation, we did not observe any statistically significant different in serum cytokines. Therefore, we do not have direct evidence of increased systemic inflammation in the MDD group; however, given that increased levels of circulating pro-inflammatory cytokines have previously been observed in subsets of MDD patients, our study may not have been powered to detect these differences. Future work perhaps specifically focusing on gastrointestinal inflammation, through markers such as fecal calprotectin or lipocalin-2, may be warranted. Finally, we cannot account for all of the complex confounding variables that may also contribute to microbiome differences between the two groups, such as differential lifestyle factors such as dietary habits or non-psychotropic medication use.

5. Conclusions

The microbiomes of American young adults with major depressive disorder were found to be significantly different from those of healthy controls. At high taxonomic levels, depressed subjects had lower levels of the phylum Firmicutes, class Clostridia, and order Clostridiales, and correspondingly higher levels of the phylum Bacteroidetes, classes Bacteroidia and Gammaproteobacteria, and order Bacteroidales. Most notably, subjects with MDD had lower levels of the families *Ruminococcaceae* (including the genera *Faecalibacterium*, *Subdoligranulum*, *Ruminococcus 1*, and *[Eubacterium] coprostanoligenes* group), *Christensenellaceae* (including the genus-level taxon *R-7* group), and *Barnesiellaceae* (including the genus *Barnesiella*). These subjects also had higher levels of the *Ruminococcaceae* genus *Flavonifractor* and the *Lachnospiraceae* genus *Sellimonas*. Additionally, we found that the majority of notable taxonomic changes in the depressed group were more pronounced in subjects with higher scores on a depressive symptom scale, although we cannot rule out the impact of psychotropic medication due to significant overlap of usage with symptom severity. Overall, our findings align with previous studies of the gut microbiota in subjects with depression, particularly that depressed subjects have lower levels of *Faecalibacterium* and higher levels of *Flavonifractor*. In general, the differences that we observed are supportive of an inflammatory state in subjects with MDD, as these subjects tended to have lower levels of butyrate-producing, anti-inflammatory bacteria such as *Faecalibacterium* and *Subdoligranulum* and higher levels of taxa previously associated with inflammatory disorders such as *Flavonifractor* and Gammaproteobacteria. Importantly, there was significant overlap in the proportions of the discriminant taxa between the control and MDD groups in most cases, aligning with previous observations that inflammation may play a role in the etiology of depression in a significant subset of patients but is neither necessary nor sufficient to cause its onset.

6. Declarations

Author contributions

RTL and PB conceptualized the study. RTL, AES, CMS, and RFLW recruited subjects and collected samples. ADR prepared the samples, analyzed data, and generated the figures. BJK performed experiments and contributed to data analysis. ADR and RTL wrote the manuscript. PB edited the manuscript.

7. Data availability

Underlying sequencing data can be found at the NCBI Short Read Archive under Project ID PRJNA591924.

8. Ethics

This study was approved by the Lifespan Institutional Review Board.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.03.026.

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