UC San Diego

UC San Diego Previously Published Works

Title

Gut microbiome in Schizophrenia: Altered functional pathways related to immune modulation and atherosclerotic risk.

Permalink

https://escholarship.org/uc/item/722278c9

Authors

Nguyen, Tanya T
Kosciolek, Tomasz
Daly, Rebecca E
et al.

Publication Date

2021

DOI

10.1016/j.bbi.2020.10.003

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Brain, Behavior, and Immunity xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Gut microbiome in Schizophrenia: Altered functional pathways related to immune modulation and atherosclerotic risk

Tanya T. Nguyen[a,b,*,1], Tomasz Kosciolek[c,d,1], Rebecca E. Daly[b], Yoshiki Vázquez-Baeza[e], Austin Swafford[e], Rob Knight[c,e,f,g,2], Dilip V. Jeste[a,c,e,h,2]

[a] Department of Psychiatry, University of California, San Diego, CA, United States
[b] Sam and Rose Stein Institute for Research on Aging, University of California, San Diego, CA, United States
[c] Department of Pediatrics, University of California, San Diego, CA, United States
[d] Małopolska Centre of Biotechnology, Jagiellonian University, Kraków, Poland
[e] Center for Microbiome Innovation, University of California, San Diego, CA, United States
[f] Department of Computer Science and Engineering, University of California, San Diego, CA, United States
[g] Department of Computer Science and Engineering, University of California, San Diego, CA, United States
[h] Department of Neurosciences, University of California, San Diego, CA, United States

ARTICLE INFO

Keywords:
Psychosis
Serious mental illness
Accelerated aging
Gut-brain axis
Microbes
Bacteria
Functional pathways
Inflammation

ABSTRACT

Emerging evidence has linked the gut microbiome changes to schizophrenia. However, there has been limited research into the functional pathways by which the gut microbiota contributes to the phenotype of persons with chronic schizophrenia. We characterized the composition and functional potential of the gut microbiota in 48 individuals with chronic schizophrenia and 48 matched (sequencing plate, age, sex, BMI, and antibiotic use) non-psychiatric comparison subjects (NCs) using 16S rRNA sequencing. Patients with schizophrenia demonstrated significant beta-diversity differences in microbial composition and predicted genetic functional potential compared to NCs. Alpha-diversity of taxa and functional pathways were not different between groups. Random forests analyses revealed that the microbiome predicts differentiation of patients with schizophrenia from NCs using taxa (75% accuracy) and functional profiles (67% accuracy for KEGG orthologs, 70% for MetaCyc pathways). We utilized a new compositionally-aware method incorporating reference frames to identify differentially abundant microbes and pathways, which revealed that *Lachnospiraceae* is associated with schizophrenia. Functional pathways related to trimethylamine-N-oxide reductase and Kdo_2-lipid A biosynthesis were altered in schizophrenia. These metabolic pathways were associated with inflammatory cytokines and risk for coronary heart disease in schizophrenia. Findings suggest potential mechanisms by which the microbiota may impact the pathophysiology of the disease through modulation of functional pathways related to immune signaling/response and lipid and glucose regulation to be further investigated in future studies.

1. Introduction

Schizophrenia is a debilitating illness of the brain and body. It is associated with cognitive and functional deficits as well as higher medical comorbidity and shortened life expectancy that limit individuals' quality and quantity of life (Casey et al., 2009). Individuals with schizophrenia are more prone to diseases associated with aging, namely cardiovascular diseases (CVD) (Hennekens et al., 2005), and exhibit age-associated physiological changes, such as inflammation, at earlier ages (Lee et al., 2017; Kirkpatrick and Miller, 2013). A growing body of literature strongly supports the hypothesis of accelerated biological aging in persons with schizophrenia (Kirkpatrick et al., 2008; Nguyen et al., 2018; Jeste et al., 2011). Understanding the mechanisms of potential accelerated aging is imperative to improving the quality and quantity of life in schizophrenia. Mounting evidence links the gut microbiome – the modifiable "second genome" consisting of trillions of diverse microbes living in the human gut – to key determinants of human health and disease (Clemente et al., 2012). The gut microbiome is critical in maintaining human physiology. It regulates many metabolic processes essential for optimal health that cannot be maintained

* Corresponding author at: University of California, San Diego, 9500 Gilman Drive #0664, La Jolla, CA 92093, United States.
E-mail address: ttn050@health.ucsd.edu (T.T. Nguyen).
[1] Co-first authors.
[2] Co-senior authors.

https://doi.org/10.1016/j.bbi.2020.10.003
Received 16 July 2020; Received in revised form 5 October 2020; Accepted 6 October 2020
0889-1591/ Published by Elsevier Inc.

ARTICLE IN PRESS

by human cells, particularly in maintaining homeostasis of host metabolism, stimulating normal immune maturation, and stabilizing the gut barrier (Carroll et al., 2009). Imbalance (or dysbiosis) of the microbiota has been associated with most aging-related diseases, including diabetes, obesity, CVD, and neurodegenerative diseases (Clemente et al., 2012). These observations have raised a possibility that the human microbiome is a key modulator of host organismal aging, which may contribute to premature morbidity and mortality in schizophrenia. Schizophrenia is also characterized by increased gut permeability (Severance et al., 2013). In this way, gut dysbiosis may contribute to increased translocation of enteric microbes into systemic circulation, thus potentially contributing to the pro-inflammatory milieu and other physiological abnormalities implicated in schizophrenia (Hsiao et al., 2013).

Research on the gut microbiome in psychiatric disorders is in early stages and the role of the gut-brain axis in schizophrenia is not fully understood. Our previous systematic reviews in this area (Nguyen et al., 2018, 2019) found a small number of studies examining differences in the gut microbiota between patients with first-episode psychosis and schizophrenia and non-psychiatric comparison subjects (NCs). Although all investigations reported beta-diversity differences between patients and controls, there was minimal consensus with regards to abundances of microbial taxa. Discrepancies may be explained, at least partly, by misinterpretations of relative abundance data due to the compositionality of microbiome data, which we sought to resolve using novel methods. The present study is an extension of our previous article (Nguyen et al., 2019) characterizing the gut microbiome in patients with chronic schizophrenia; it includes participants from the previous study. With an expanded cohort ($n = 96$), we build upon our previous results ($n = 50$) and incorporate new analyses on functional pathways, which have not been previously reported in any subjects. The objectives of this study were three-fold. 1) We evaluated differential abundance using Songbird, a newly-developed compositionally-aware method for differential abundance testing (Morton et al., 2019). This approach is more robust than previous methods and may help reveal more reproducible results and lead to more stable inferences of compositional change. 2) We also evaluated the predicted functional potential of the microbial community using PICRUSt2 (Langille et al., 2013; Douglas et al., 2019). Understanding the genetic potential of the intestinal ecosystem may be more important and robust across different studies and cohorts than simply knowing the taxonomic identity of microbes present, particularly in elucidating potential pathways and mechanisms by which the gut microbiome influences downstream clinical outcomes. 3) Finally, using supervised learning (Random Forests models), we evaluated the microbiome as a diagnostic predictive tool by constructing taxonomy-based and function-based classifiers.

Based on previous studies (Nguyen et al., 2019; Shen et al., 2018; Zheng et al., 2019), we predicted that beta-diversity, but not alpha-diversity, of microbial taxa and predicted functional pathways would be significantly different between schizophrenia and NC groups. Furthermore, consistent with the theoretical framework of accelerated biological aging in schizophrenia, we also hypothesized that schizophrenia would be characterized by alterations in functional pathways involved in immune modulation and cardiovascular risk. We also anticipated that alterations in microbiota function would be associated with indicators of inflammation and CVD risk, in schizophrenia.

2. Materials and methods

2.1. Participants

Participants were recruited from the greater San Diego area through the University of California San Diego (UCSD). The research protocol was approved by the UCSD Human Research Protections Program, and all participants provided written informed consent prior to participation. The sample included 48 subjects with schizophrenia and 48 matched NCs between the ages of 27 and 76 years. Participants with schizophrenia were diagnosed based on the Structured Clinical Interview for the DSM-IV-TR (SCID) (First et al., 2002). NCs were screened for major neuropsychiatric disorders using the Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998) and excluded if they had a past or present diagnosis of a major neuropsychiatric illness. Exclusion criteria were: other current major DSM-IV-TR Axis I diagnoses; alcohol or other substance (other than tobacco) abuse or dependence within 3 months prior to enrollment; diagnosis of dementia, intellectual disability disorder, or a major neurological disorder; or any medical disability that interfered with a subject's ability to complete study procedures. Additional details regarding recruitment and subject selection have been previously reported (Nguyen et al., 2019).

To account for potential methodological differences that might bias results and obscure biologically meaningful compositional differences (Lozupone et al., 2013; Walters et al., 2014) and to control for clinical factors and known major drivers of microbiome changes (McDonald et al., 2018), NCs were matched to schizophrenia subjects on the same sequencing plate after initial recruitment. Following a stable matching algorithm, for each SZ subject we found the nearest matching NC neighbor based on age, sex, race, body mass index (BMI), and history of antibiotic use, requiring that the sample must come from the same sequencing plate.

2.2. Sociodemographic and clinical assessment

Sociodemographic characteristics (i.e., age, sex, race/ethnicity, current smoking status) and illness-related factors (i.e., age of onset, duration of illness, antipsychotic medication dosages) were obtained through participant interview and review of the available medical records. Antipsychotic medication dosages were converted to World Health Organization (WHO) defined daily dose (Organization WH, 2017). BMI was calculated based on height and weight (kg/m^2). Participants were interviewed by trained staff and completed the following standardized assessments. Positive and negative psychiatric symptoms were evaluated using interviewer-administered Scales for Assessment of Positive Symptoms and Negative Symptoms (SAPS, SANS) (Andreasen, 1983; AndreasenOthers., 1984). Depression was assessed using the Patient Health Questionnaire (PHQ-9) (Kroenke et al., 2001). Health-related quality of life and functioning was evaluated using the physical and mental health component scores from the Medical Outcomes Study 36-item Short Form (SF-36) (Ware and Sherbourne, 1992). Medical comorbidity was measured with the total score and severity index from the Cumulative Illness Rating Scale (CIRS) (Parmelee et al., 1995). The Framingham 10-year Coronary Heart Disease (CHD) Risk Score was calculated according to Wilson et al (Wilson et al., 1998).

2.3. Inflammatory biomarker assays

A subsample of participants was randomly selected to provide fasting blood samples for testing of specific biomarkers. Of these individuals, a greater proportion was from the schizophrenia group ($p < 0.001$) and of non-Caucasian race ($p < 0.047$). Otherwise, the subsample did not significantly differ from the overall sample on age, gender, BMI, medical comorbidity, smoking status, psychiatric symptomatology, or antipsychotic medication use ($ps > 0.05$). Based on prior empirical literature (Lee et al., 2017; Fernandes et al., 2016; Frydecka et al., 2015), we focused on three pro-inflammatory markers that have been observed to be linked with schizophrenia (high-sensitivity C-reactive protein [hs-CRP], tumor necrosis factor [TNF]α, and interleukin [IL]-6) and explored relationships with an anti-inflammatory cytokine (IL-10). Plasma TNFα, IL-6, and IL-10 were quantified using the Meso Scale Discovery (MSD) MULTI-SPOT® Assay System (MSD, Rockville, MD, USA) and analyzed on a SECTOR Imager 2400 instrument (MSD). Samples were run in duplicates, using V-PLEX

Human Biomarker panels (Catalog no. K151A0H-2). Plasma hs-CRP was measured with MSD enzyme-linked immunosorbent assay (ELISA). Intra-assay variability and inter-assay variability was < 10% for all assays.

2.4. Fecal sample collection and processing

Participants collected fecal samples using home collection kits (BD SWUBE Dual Swab Collection System; BD Worldwide) and returned them via mail in a self-addressed envelope. Returned samples were immediately frozen and stored at −80 °C until aliquoting into 96 well plates for DNA extraction. DNA extraction and 16S rRNA amplicon sequencing were completed using the Earth Microbiome Project standard protocols (McDonald et al., 2018; Thompson et al., 2017). In brief, DNA was extracted using the Qiagen MagAttract PowerSoil DNA kit (Marotz et al., 2017), followed by PCR amplification on the V4 region of the 16S rRNA gene with unique reverse barcoded primers and sequenced on Illumina MiSeq and HiSeq 4000 platforms, yielding paired-end, 150 base-pair reads, with a minimum of 7,872 (median 47,615) reads per sample (Caporaso et al., 2012; Walters et al., 2016).

2.5. 16S data processing and analysis

The raw sequencing data were processed using QIIME 2 version 2019.7 (Caporaso et al., 2010; Bolyen et al., 2019). Raw sequences were demultiplexed and processed using Deblur to generate amplicon sequence variants (ASVs) (Amir et al., 2017) and previously recognized bloom sequences were removed (Amir et al., 2017). Deblur ASVs were inserted into the Greengenes 16S rRNA gene tree using SEPP via q2-fragment-insertion (McDonald et al., 2012; Janssen et al., 2018), and taxonomy was assigned using UCLUST (Edgar, 2010). The output feature table was rarefied to 7,000 sequences per sample. PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states, version 2) was performed to predict functional potential of microbial communities, based on metagenomes inferred from 16S data (Langille et al., 2013; Douglas et al., 2019). PICRUSt2 predictions were made based on the following gene family databases: Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) (Kanehisa et al., 2012), Enzyme Classification (EC) numbers, and the MetaCyc Metabolic Pathway Database (Caspi et al., 2018).

Primary measures of the gut microbiome included abundances of 1) microbial taxa (e.g., ASVs) and 2) functional pathways. For each, core diversity metrics of alpha-diversity (within-sample) and beta-diversity (between-samples) were calculated. Metrics of alpha-diversity included number of observed features, Shannon diversity index (Shannon, 1948), and Faith's Phylogenetic Diversity (PD) (Faith, 1992). Beta-diversity was calculated using unweighted UniFrac (Lozupone and Knight, 2005; Lozupone et al., 2007) and Bray-Curtis dissimilarity (Bray and Curtis, 1957) for taxa, and Jaccard distance (Jaccard, 1912) for predicted functional pathways. Output matrices were ordinated using principal coordinate analysis (PCoA) and visualized using EMPeror (Vázquez-Baeza et al., 2013).

2.6. Statistical analysis

Participant sociodemographic and clinical characteristics were summarized and analyzed using paired-samples *t* tests and McNemar χ^2 tests for continuous and discrete variables, respectively. Analyses of microbiome 16S sequencing data were performed in QIIME 2. A two-sided alpha level of $p < 0.05$ was used to determine statistical significance. The Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) was used to control false discovery rate (FDR) at alpha = 0.05 to account for multiple comparisons within each set of analyses.

2.6.1. Diversity analysis

Alpha-diversity metrics were compared using nonparametric Wilcoxon signed-rank tests. Beta-diversity distances between samples were compared with PERMANOVA (Anderson, 2001) with 999 permutations.

2.6.2. Differential abundance analysis

Differential abundance testing of microbial taxa and functional pathways were first performed using ANCOM (Mandal et al., 2015). We used features identified by ANCOM to seed Songbird to further examine differential abundance (Morton et al., 2019). Songbird is a recently developed method that uses reference frames (or balances) to account for the compositional nature of microbiome data. By comparing log-ratios of features, this method circumvents bias introduced by unknown total microbial loads. We employed Songbird to estimate the relative differentials of microbial taxa and functional pathways based on disease status (schizophrenia vs. NC). Relative differentials were then ranked with respect to diagnosis (i.e., most associated with schizophrenia vs. NC groups) and visualized using Qurro (Fedarko et al., 2019). Balances were created to determine differential abundance, relative to reference frame, and compared using Wilcoxon signed-rank tests. Spearman's correlations were performed to explore associations of log-ratios of functional pathways with biomarkers of inflammation and CVD risk.

2.6.3 Random forests analysis. We used supervised learning Random Forests models (Breiman, 2001) to classify samples according to diagnosis (i.e., schizophrenia or NC). For ASV models, data were divided into 80%-20% training-test sets and prior to training the model, the data were imported into Calour (Xu et al., 2019), normalized to 10,000 reads per sample and, in order to limit the number of features in the model, low abundance features (20 reads across all samples or less) were clustered together based on Euclidean distance hierarchical clustering. In PICRUSt2 models, for MetaCyc pathways, we normalized the data to 5,000,000 reads per sample, since there is approximately 100x more pathways than ASVs, and filtered features with < 10,000 occurrences across all samples; for KEGG orthologs, we normalized the data to 50,000,000 reads per sample, due to approximately 1,000x more KEGG orthologs than ASVs, and filtered features with < 1,000,000 occurrences across all samples. Hyperparameter optimization of the model was performed using the grid search method, as implemented in Python scikit-learn package (Bokulich et al., 2018; Pedregosa et al., 2011; Virtanen et al., 2020). ROC curves were generated from the stratified 5-fold cross-validation results and the confusion matrices were calculated on the basis of the best model obtained from cross-validation. The best model was selected on the basis of accuracy score.

3. Results

Participant demographic and clinical characteristics are presented in Table 1. As expected, the schizophrenia group had worse psychiatric symptoms, lower levels of physical/mental well-being, higher rates of smoking, and greater medical comorbidity. Although schizophrenia and NC groups did not differ in mean levels of blood-based inflammatory biomarkers, a significantly greater proportion of patients with schizophrenia exhibited hs-CRP levels ≥ 2 mg/L, a level above which is associated with increased risk of major cardiovascular events (Carrero et al., 2019).

3.1. Microbiota analysis

3.1.1. Diversity patterns

Schizophrenia and NC groups did not differ on any assessed measure of alpha-diversity (Table S1). There were significant beta-diversity differences between schizophrenia and NC groups using unweighted UniFrac and Bray-Curtis (Table 2). PCoA of unweighted UniFrac and Bray-Curtis distances showed that schizophrenia and NC groups formed

ARTICLE IN PRESS

Table 1
Demographic and Clinical Characteristics of Subjects.

	Non-Psychiatric Comparison(n = 48)	Schizophrenia(n = 48)	t or χ^2	p
Age (years)	54.1 (12.6)	53.2 (10.3)	N/A[1]	–
Gender [n (% female)]	19 (40%)	19 (40%)	N/A[1]	–
Race [n (% Caucasian)	37 (77%)	31 (65%)	N/A[1]	–
BMI	28.5 (5.9)	31.8 (6.7)	N/A[1]	–
BMI classification[2] [n (%)]				
Normal	10 (21%)	9 (19%)	N/A[1]	–
Overweight	24 (50%)	11 (23%)		
Obese	14 (29%)	28 (58%)		
Antibiotic use [n (% in past year)]	12 (25%)	12 (25%)	N/A[1]	–
Current smoking status [n (% smoker)]	2 (4%)	16 (33%)	14.6	0.007
Age of illness onset (years)	–	23.5 (9.1)	–	–
Illness duration (years)	–	30.3 (11.0)	–	–
Current antipsychotic use [n (%)]				
On	–	40 (83%)	–	–
Off		8 (17%)		
Antipsychotic type [n (%)]				
Atypical	–	23 (57%)	–	–
Typical		6 (15%)		
Both		11 (28%)		
Antipsychotic daily dosage[3]	–	2.06 (2.20)	–	–
SAPS Total Score	0.50 (0.8)	4.58 (3.5)	−3.72	0.003
SANS Total Score	0.58 (0.9)	4.81 (4.3)	−3.84	0.003
PHQ-9 Depression Score	3.24 (3.9)	7.49 (5.5)	−3.87	< 0.001
SF-36 Mental Component	52.2 (8.3)	43.6 (12.8)	2.97	0.005
SF-36 Physical Component	52.3 (8.4)	41.9 (10.8)	4.81	< 0.001
CIRS Total Score	3.27 (3.6)	6.74 (3.1)	−2.03	0.07
CIRS Severity Score	0.95 (0.8)	1.63 (0.4)	−2.87	0.02
Framingham 10-year CHD % Risk	0.083 (0.06)	0.10 (0.07)	−1.521	0.19
Medical diagnoses				
Diabetes [n (% with)]	0 (0%)	16 (50%)	5.46	< 0.001
Heart Disease [n (% with)]	0 (0%)	8 (17%)	2.31	< 0.001
Hypertension [n (% with)]	6 (50%)	32 (67%)	1.15	0.052
Inflammatory biomarkers[4]				
hs-CRP (mg/L)	1.38 (2.02)	3.18 (3.80)	−1.63	0.178
hs-CRP clinical high risk[5] [n (%)]	2 (4%)	16 (33%)	13.4	< 0.001
TNFα (pg/mL)[6]	2.79 (1.17)	2.88 (1.66)	0.09	0.93
IL-6 (pg/mL)[6]	0.59 (0.12)	1.10 (0.73)	−1.47	0.16
IL-10 (pg/mL)[6]	0.32 (0.04)	0.42 (0.23)	−0.42	0.68

BMI = body mass index; CHD = coronary heart disease; hs-CRP = high sensitivity C-reactive protein; IL = interleukin; PHQ-9 = Patient Health Questionnaire; SANS = Scale for the Assessment of Negative Symptoms; SAPS = Scale for the Assessment of Positive Symptoms; TNFα = tumor necrosis factor alpha; WHO = World Health Organization

[1] Comparison not applicable as groups were matched on these variables.

[2] World Health Organization (WHO) classification

[3] WHO defined daily dose

[4] Values for statistical tests based on log-transformed values

[5] hs-CRP ≥ 2 mg/L; higher risk of major adverse cardiovascular event (Carrero Juan Jesus, Andersson Franko Mikael, Obergfell Achim, Gabrielsen Anders, Jernberg Tomas. hsCRP Level and the Risk of Death or Recurrent Cardiovascular Events in Patients With Myocardial Infarction: a Healthcare-Based Study. J Am Heart Assoc., 2019)

[6] Independent t-tests were performed, as not all subjects in pairs had inflammatory biomarker data

Table 2
Differences in Microbial Beta-Diversity Indicators by Diagnostic Group and Other Demographic and Clinical Variables.

Variable	Unweighted UniFrac			Bray-Curtis Dissimilarity		
	pseudo-F	p	$q^{\dagger}$	pseudo-F	p	q
Diagnosis	2.41	0.001	0.010*	2.74	0.002	0.010*
Sex	1.65	0.013	0.022*	1.88	0.007	0.018*
BMI category	1.39	0.011	0.022*	1.40	0.034	0.043*
Smoking status	1.79	0.005	0.167	1.54	0.034	0.043*
Current antipsychotic medication use	0.92	0.60	0.67	0.77	0.826	0.826

† Adjusted p-value controlling for false discovery rate using Benjamini-Hochberg procedure

* $q < 0.05$.

BMI = body mass index.

distinct clusters (Fig. 1). Additionally, schizophrenia subjects demonstrated greater variability across the PCoA space, compared to NCs who were more tightly clustered (within-group distance comparison; unweighted UniFrac $p < 0.001$; Bray-Curtis $p < 0.001$). Across diagnostic groups, significant beta-diversity differences were also observed for sex, BMI category, and smoking status (Table 2). Within the schizophrenia group, there were no beta-diversity differences between subjects on versus off antipsychotic medication.

3.1.2. Differential abundance

Differential abundance testing using ANCOM identified two taxa from family *Lachnospiraceae* to be significantly different between schizophrenia and NC groups (W = 647, 688). The W value is used to evaluate the null hypothesis that the two groups have no significant difference; the higher the W value, the more significantly the taxon differs across groups. Songbird was then employed to identify taxa that were most positively and negatively associated with diagnosis, and Qurro was used to display the sorted differential rankings associated

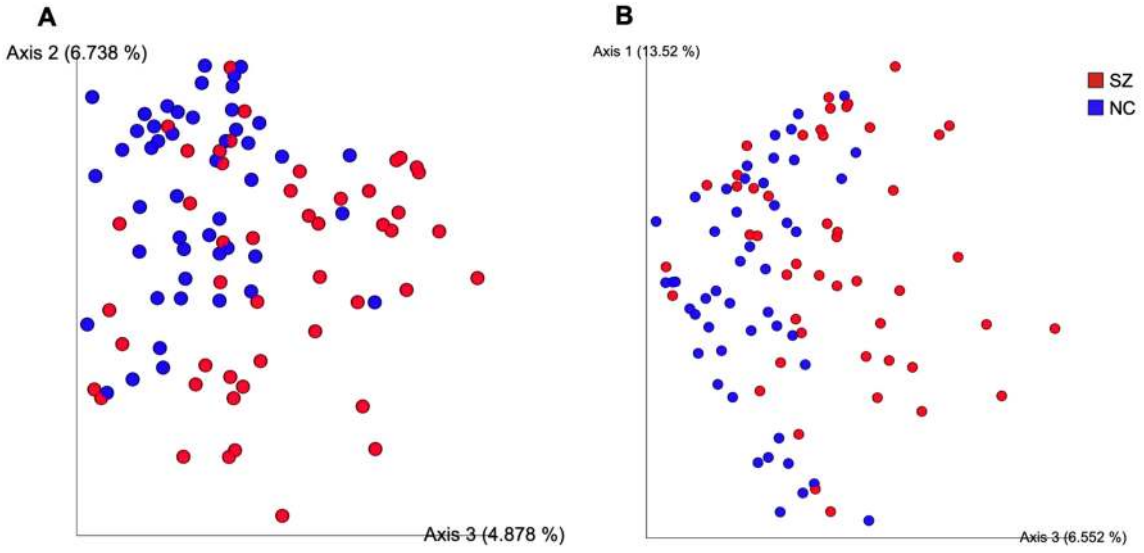


Fig. 1. Beta-diversity of microbial taxa was significantly different between schizophrenia and NC groups. PCoA plots are a multivariate reduction method to depict beta-diversity distance matrices of microbial taxa between samples. Each point represents an individual subject. (**A**) Unweighted UniFrac measures the presence or absence of unique branch lengths in a phylogenetic tree, while (**B**) Bray-Curtis is a non-phylogenetic method that considers relative abundance. Both unweighted UniFrac (**A**) and Bray-Curtis (**B**) distance metrics show a high degree of separation between participants with schizophrenia (red) compared to NC participants (blue) along Axis 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with schizophrenia vs. NC groups, as determined by Songbird (Fig. 2**A**). The differential microbial ranks are listed in Table S2. A log-ratio was computed with the sequences associated with the family *Lachnospiraceae* (as identified by ANCOM) in the numerator, compared to the top-20 ranked taxa (i.e., taxa most associated with NCs) in the denominator as a reference frame. The difference in this log-ratio was compared between groups, and across this balance, *Lachnospiraceae* was significantly higher in the schizophrenia group compared to NC group ($p = 0.002$) (Fig. 2**B**).

3.2. Functional analysis

To identify potential genetic functional differences between microbial communities present, PICRUSt2 was performed to predict microbial metagenomes, which can reveal the specific metabolic and/or biological functions of the gut microbiome.

3.2.1. Diversity patterns

There were no group differences in the alpha-diversity of KEGG

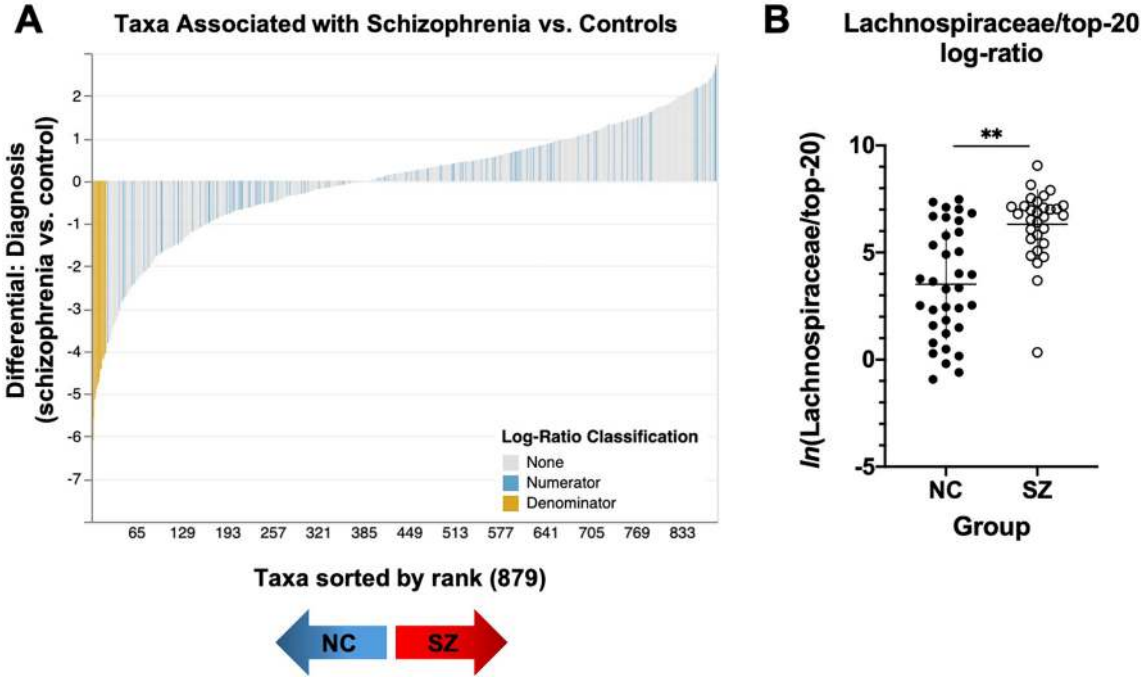


Fig. 2. Differential rankings of taxa associated with diagnosis. (**A**) Qurro visualization displaying the sorted differential rankings of taxa associated with schizophrenia vs. NC groups, as determined by Songbird. Left on the x-axis indicates relative over-expression in NCs, right indicates relative over-expression in schizophrenia. Sequences associated with family *Lachnospiraceae* are highlighted in blue; the top-20 ranked taxa are highlighted in yellow. (**B**) The log-ratio of *Lachnospiraceae* to the top-20 ranked taxa is significantly increased in schizophrenia, compared to NCs ($Z = -3.072$, $p = 0.002$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

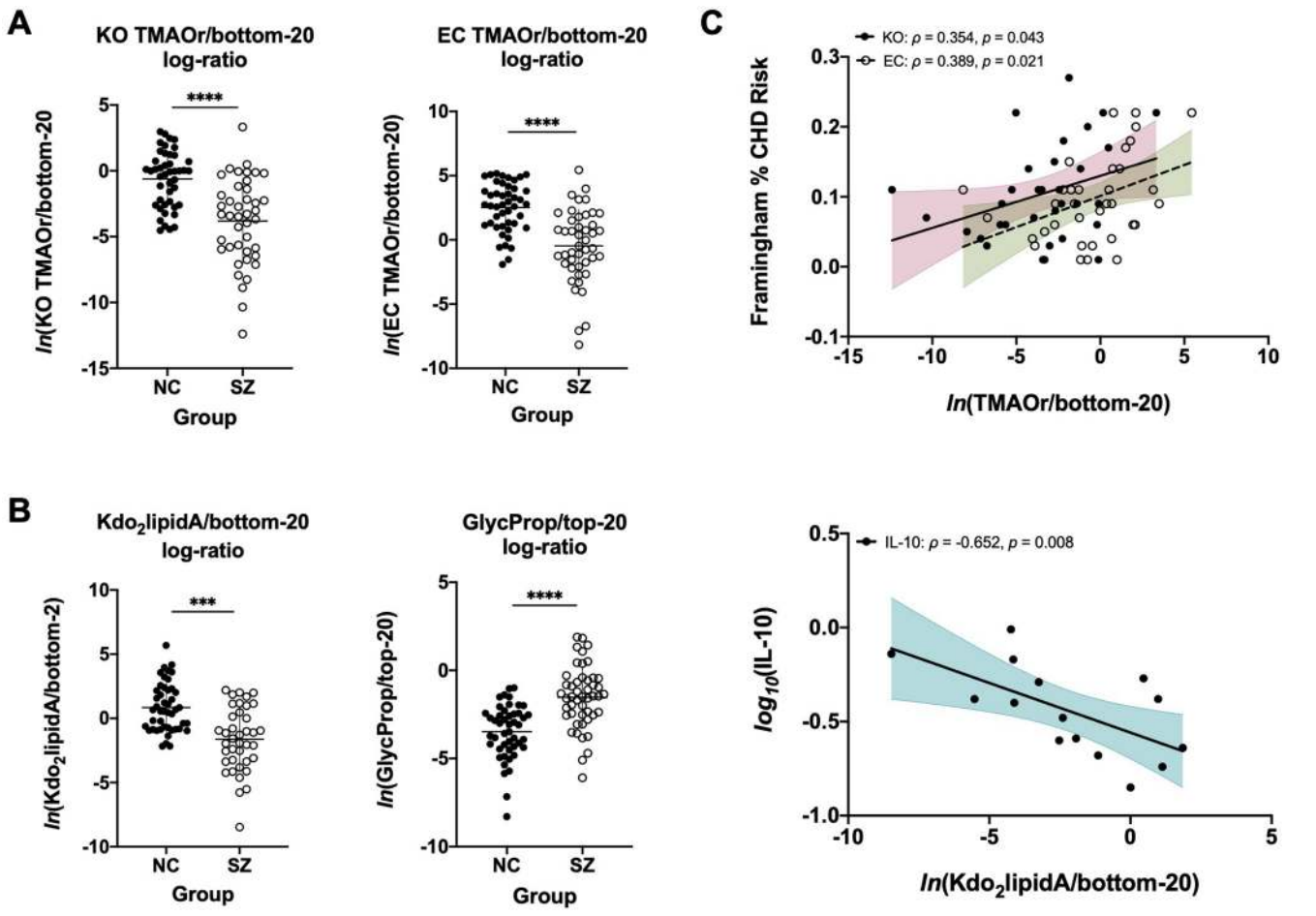


Fig. 3. Three predicted functional pathways were differentially abundant between schizophrenia and NC groups: trimethylamine-N-oxide (TMAO) reductase, glycerol degradation to 1,3-propanediol, and Kdo_2-lipid A biosynthesis. (**A**) The log-ratios of TMAO reductase (TMAOr), relative to the bottom-20 pathways (i.e., pathways most associated with schizophrenia), were significantly lower in schizophrenia, compared to NCs. K07821: Z = -3.80, $p < 0.001$, $q < 0.001$; EC 1.7.2.3: Z = -4.19, $p < 0.001$, $q < 0.001$. (**B**) The log-ratio of glycerol degradation to 1,3-propanediol pathway (GlycProp), relative to the top-20 pathways (i.e., most associated with NCs), was significantly increased in schizophrenia, whereas the log-ratio of Kdo_2-lipid A biosynthesis pathway, relative to the bottom-20 pathways, was significantly decreased in schizophrenia group, compared to NCs. GOLPDLCAT-PWY: Z = -4.36, $p < 0.001$, $q < 0.001$; KDO-NAGLIPASYN-PWY: Z = -3.33, p = 0.001, $q < 0.001$ (**C**) Greater log-ratios of TMAOr/bottom-20 were associated with greater Framingham Risk Scores in schizophrenia, and greater log-ratios of Kdo_2-lipid A/bottom-20 was correlated with decreased levels of IL-10.

orthologs, EC numbers, or MetaCyc pathways ($ps > 0.08$). Beta-diversity was significantly different between schizophrenia and NC groups for KEGG orthologs (Jaccard pseudo-F = 2.41, p = 0.008, q = 0.012) and EC numbers (Jaccard pseudo-F = 2.49, p = 0.007, q = 0.012), but not MetaCyc pathways (Jaccard pseudo-F = 1.28, p = 0.252, q = 0.252).

3.2.2. Differential abundance

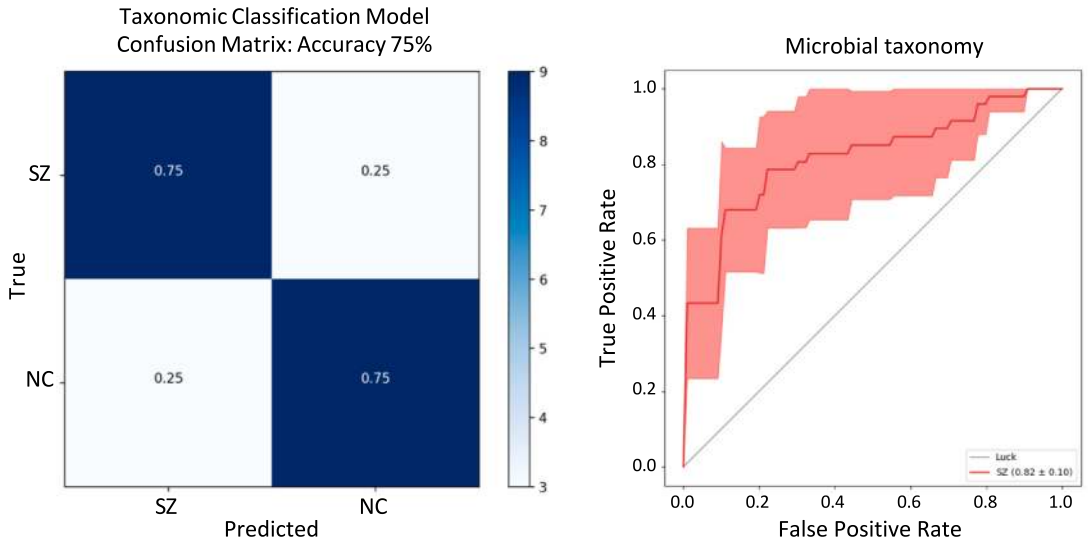
ANCOM revealed three predicted functional pathways to be differentially abundant between groups: trimethylamine-N-oxide (TMAO) reductase (KO W = 3725; EC W = 1243), glycerol degradation to 1,3-propanediol (W = 109), and Kdo_2-lipid A biosynthesis (W = 76). The differential ranking of functional pathways associated with diagnosis were generated using Songbird. The differential ranks for KEGG orthologs, EC numbers, and MetaCyc pathways are listed in Table S3. The log ratios of the above-identified pathways to either the top-20 or bottom-20 ranked pathways were computed and compared between groups. Log-ratios with TMAO reductase as the numerator, relative to bottom-20 pathways, were significantly lower in the schizophrenia group compared to the NC group ($q < 0.001$) (Fig. 3**A**). The log-ratio of glycerol degradation to 1,3-propanediol pathway, relative to the top-20 pathways, was significantly increased in the schizophrenia group, whereas the log-ratio of Kdo_2-lipid A biosynthesis pathway, relative to the bottom-20, was significantly decreased in the schizophrenia group, compared to NCs ($q < 0.001$) (Fig. 3**B**).

The extant literature suggest links between TMAO and Kdo_2-lipid A with CVD outcomes and immune responses, respectively (Zeisel and Warrier, 2017; Wang et al., 2015), which are both related to the hypothesis of accelerated aging in schizophrenia. Spearman's correlations were performed to examine the association of these functional pathways with age, Framingham Risk Score, and inflammatory biomarkers (Fig. 3**C**). Log-ratios of TMAO reductase/bottom-20 were positively correlated with Framingham Risk Score. The log-ratio of Kdo_2-lipid A/bottom-20 was negatively correlated with IL-10. No significant correlations were observed with age, hs-CRP, IL-6, or TNFα in schizophrenia. No relationships between pathways and clinical variables were observed in the NC group ($ps > 0.13$).

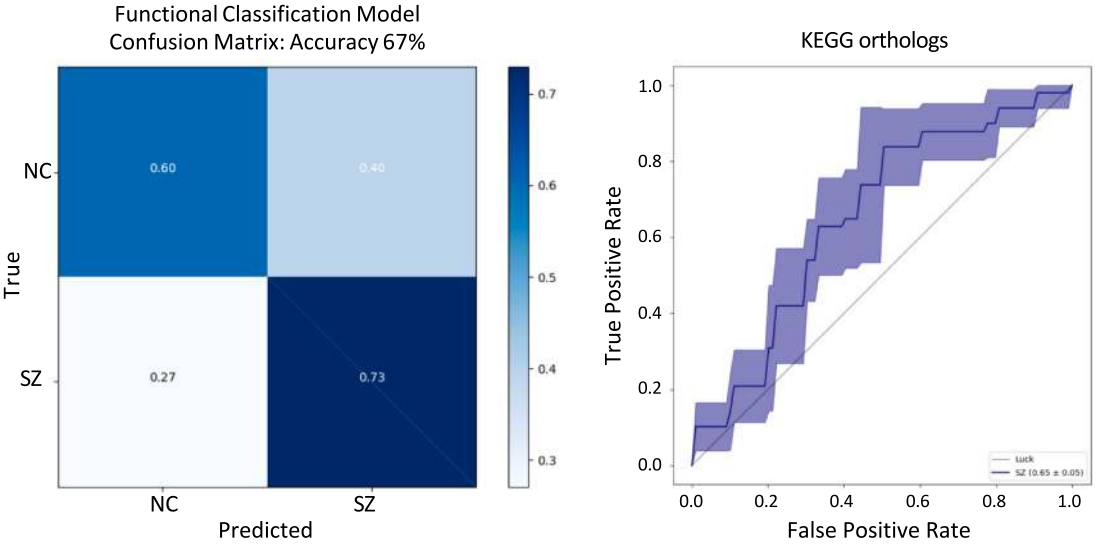
3.3. Random forests classifier

To evaluate the ability of the microbiome to classify diagnostic groups, we used Random Forests models to classify groups using microbial composition (ASVs) and functional profiles (KEGG orthologs and MetaCyc pathways) as key input features (Fig. 4). The model built using microbial taxonomy differentiated participants with schizophrenia from NCs with 75% accuracy. Feature importance scores were

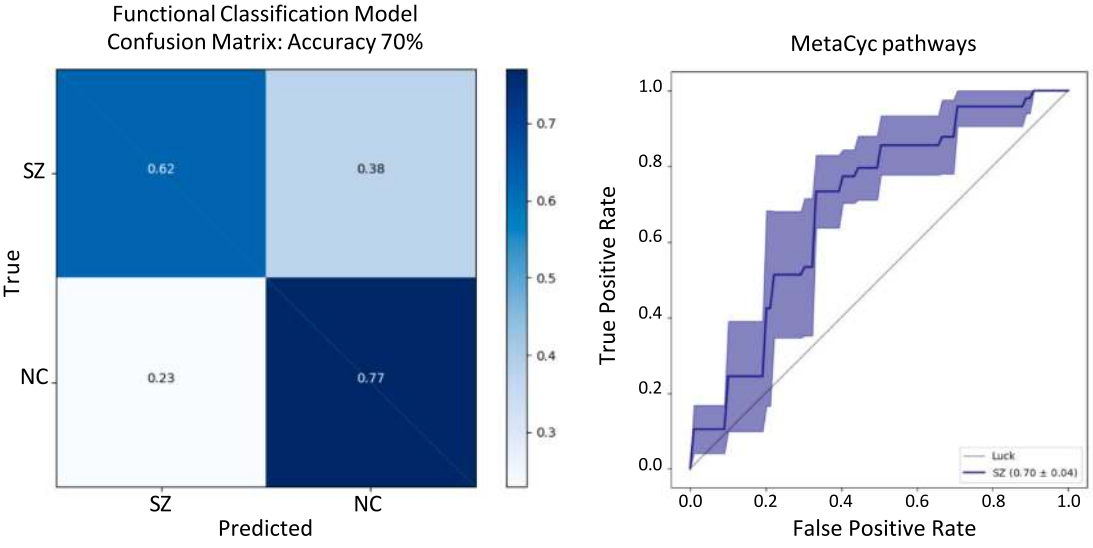
A



B



C



(caption on next page)

Fig. 4. Accuracy of supervised Random Forests classification models. (**A**) Microbial classification predicted schizophrenia vs. NCs with 75% accuracy. Area under the ROC curve was 0.82, indicating good classification accuracy. Functional classification predicted diagnosis with (**B**) 67% accuracy for KEGG orthologs and (**C**) 70% for MetaCyc pathways. All ROC curves were produced from stratified 5-fold cross-validation results, while the confusion matrices were generated from the best model obtained in cross-validation.

all < 2%, indicating that no single (or group of) microbial feature(s) contributed to model performance in a significant way. This suggests that many different features and their unique combination are jointly responsible for the overall model performance. Functional classification models were built using PICRUSt2 data. The accuracy of supervised classification was 67% for KEGG orthologs (0.65 AUC) and 70% for MetaCyc pathways (0.70 AUC). Similar to taxonomic classification models, feature importance scores from functional models were < 5%.

4. Discussion

Consistent with our hypotheses, we found significant beta-diversity differences in both microbial composition and functional potential between individuals with schizophrenia and NCs. On the other hand, alpha-diversity of microbial taxa and functional pathways were not different between groups. Using a novel compositionally-sound method to conceptualize and interpret differential abundance, we found that the family *Lachnospiraceae* was associated with schizophrenia. One of the most notable findings of this study is that pathways related to TMAO reductase and Kdo_2-lipid A biosynthesis were altered in schizophrenia, indicating that these enzymes and pathways may have clinical significance and, if verified in independent studies, may prove to be useful markers of disease. Also as hypothesized, we observed that these functional profiles were associated with decreased anti-inflammatory cytokine IL-10 and increased risk for CVD. This study extends our previous report (Nguyen et al., 2019) and presents new findings on microbial functional potential and pathways in this cohort of patients with chronic schizophrenia.

Our findings regarding community-level differences in microbiota composition is consistent with previous studies in schizophrenia (Nguyen et al., 2019; Shen et al., 2018; Zheng et al., 2019; Zhu et al., 2020). Beta-diversity findings are further reinforced by random forests analysis, which revealed that microbial taxa can predict patients with schizophrenia from NCs with 0.82 AUC. Feature importance scores from our model did not reveal any microbial features that strongly differentiated groups, despite the strong overall accuracy of the model. This suggests that no single microbial feature was responsible for model performance, but rather the unique distribution of all taxa in relation to one another are most associated with schizophrenia. Despite differences in beta-diversity of microbes, individuals with schizophrenia and NCs did not differ in terms of within-sample alpha-diversity. It typically observed that low alpha-diversity is a hallmark of dysbiosis and represents “worse” health (Yatsunenko et al., 2012). However, the literature on alpha-diversity in psychiatric populations has been mixed (e.g., no differences (Nguyen et al., 2019; Shen et al., 2018; Coello et al., 2019; Painold et al., 2019; Zheng et al., 2016; Naseribafrouei et al., 2014; Chen et al., 2018), decreased (Zheng et al., 2019; Huang et al., 2018; Ma et al., 2020), and increased (Zhu et al., 2020; Jiang et al., 2015). A recent *meta*-analysis of the gut microbiota in major depressive disorder revealed no difference in alpha-diversity between patients and NCs (Sanada et al., 2020).

The transdisciplinary nature of microbiome research brings challenges to reproducibility and replicability (Schloss, 2018), particularly in the field of clinical psychiatric research. The microbiome revolution has opened new frontiers for examining host-microbe associations in the context of understanding psychiatric disorders, leading to an increase of studies investigating microbial abnormalities in schizophrenia and related psychotic disorders. However, such rapid expansion has pitfalls, as various research groups seek to incorporate microbiome collection and analysis into ongoing clinical studies, employing varied experimental techniques at hand. Technical differences (e.g., DNA extraction methods, PCR primers, sequencing platforms, bioinformatic pipelines, taxonomy databases) can produce systematic biases that can obscure biologically meaningful results (Lozupone et al., 2013; Walters et al., 2014; Forslund et al., 2015). Systematic variation is especially problematic when the effects of a biological parameter are expected to be subtle and makes replication and integration of findings difficult, if not impossible (Debelius et al., 2016). Small sample sizes and lack of determination of within-individual sample variation are among other pitfalls of the current literature. Our systematic review in this area (Nguyen et al., 2019) found that, among a handful of studies examining microbiota differences between patients with first-episode psychosis and schizophrenia and healthy controls, there was minimal consensus with regards to alpha-diversity patterns, relative abundance, or directionality of differences in taxa. Nowhere is this problem more apparent than in differential abundance testing. Across these studies (Nguyen et al., 2019; Shen et al., 2018; Zheng et al., 2019), over 130 taxa were observed to be different with little consistency across investigations.

Differential abundance analysis is controversial throughout microbiome research, and there is widespread misconception about how to interpret microbial abundance (Morton et al., 2019). Data from next-generation high-throughput sequencing methods are compositional, meaning that abundance information is relative to the biological specimen itself, and contains information about the relationship between parts (e.g., proportions or probabilities) (Aitchison, 1986; Pawlowsky-Glahn et al., 2015). Thus, the reporting and interpretation of relative abundance data as absolute differences or changes can lead to misinterpretations of microbial community structures, as the increase of one taxon inevitably leads to the concurrent decrease of others. Since the changes of components are mutually dependent, high false discovery rates occur when compositional data are analyzed using traditional statistical methods – a fact that is frequently swept under the rug in microbiome studies (Knight et al., 2018). Instead, log ratios are a preferable way to examine differences within compositional datasets. Ratio transformations of data capture relationships between the features, and, importantly, these ratios are the same whether the data are represented as counts or proportions. This method allows for a more robust biological understanding of microbial contributions to schizophrenia. We performed ANCOM, a compositional approach that performs statistical test based transformed by an additive log ratio (Mandal et al., 2015), followed by Songbird, which uses reference frames to establish microbial composition measurement standards (Morton et al., 2019). Using this approach, we found that the log-ratio with *Lachnospiraceae* in the numerator and the top-20 ranked taxa associated with NCs in the denominator was higher in individuals with schizophrenia compared to NCs, indicating that the ratio of this taxa may have clinical significance and, if verified in independent studies, may prove to be a useful marker of the disorder. *Lachnospiraceae* is one of the most abundant families from the phylum Firmicutes found in the gut and has been associated with the production of butyrate (Biddle et al., 2013), a short chain fatty acid associated with anti-inflammatory effects (Pryde et al., 2002). Results relying on relative abundance information for this family have been mixed. Prior studies of affective disorders have reported increased levels of *Lachnospiraceae* in patients with MDD, compared to healthy controls (Zheng et al., 2016), and in patients with bipolar disorder on atypical antipsychotic medications, compared to patients not on atypical antipsychotics (Flowers et al., 2017). A majority of participants on antipsychotic medication in this sample were taking atypical antipsychotics (either alone or with typical antipsychotics), which may have contributed to the finding of increased

ARTICLE IN PRESS

Lachnospiraceae. On the other hand, *Lachnospiraceae* has also been found to be depleted in schizophrenia and positively correlated with psychosis symptom severity (Zheng et al., 2019). A recent systematic review (Vindegaard et al., 2020) noted that the most consistent finding when pooling results from affective and psychotic disorders is lower levels of *Lachnospiraceae*. It is possible that this taxon may be a trans-diagnostic marker of psychiatric disease, perhaps related to common mood symptoms or medications across disorders. Discrepant results, with regards to directionality, may be due to differences in differential abundance methods. Thus, the method currently presented holds much promise for resolving outstanding inconsistencies across previous reports by re-analyzing those datasets using reference from to make more stable inferences of compositional differences. It is critical to use appropriate statistical tools for data analyses and to continuously benchmark analytic methods in order to better understand their strengths and limitations. It is beyond the scope of the current paper to benchmark our methods against others. Instead, we refer the reader to other published studies, including benchmarking, that highlight that compositionally-aware methods are more accurate and robust with regards to microbiome data (Morton et al., 2019; Gloor et al., 2017).

Differences between differential abundance and random forests findings, which did not identify any microbial features that strongly differentiated groups, may be explained by several reasons. Generally speaking, important features in a random forests model (i.e., taxa highly contributing to a model's performance) should be differentially abundant, but differentially abundant features may not necessarily be important in the random forests model. First, differential abundance testing examines ratios of microbes, whereas the random forests model considers normalized counts. Second, rare features may be differentially abundant, but ubiquitous features are important to the model. Third, the overall microbiome composition drives diagnotic prediction in random forests models, not any single of several taxonomic features; on the other hand, in differential abundance testing, we are looking for ratios of taxa that are different between the groups.

PICRUSt2 analysis revealed broad community functional differences between individuals with schizophrenia and NCs, and functional classification using random forests predicted diagnosis with good accuracy. Similar to taxonomic data, no differences in alpha-diversity of KEGG orthologs, EC numbers, or MetaCyc pathways were observed between groups. However, functional pathways related to TMAO reductase and Kdo_2-lipid A biosynthesis were associated with NCs. In humans, TMAO is formed from trimethylamine, which is a byproduct of the bacterial metabolism of dietary choline in the intestine (Zeisel and Warrier, 2017). TMAO modulates lipid and glucose homeostasis (Warrier et al., 2015) and has been associated with a number of chronic diseases, most notably CVD and diabetes (Zeisel and Warrier, 2017). In mouse models, TMAO represents the end of a complex dietary phosphatidylcholine–choline metabolic pathway involving the gut microbiome that contributes to the pathogenesis of atherosclerotic coronary artery disease (Wang et al., 2011). Similarly, TMAO induces glucose intolerance and insulin resistance in mice fed a high fat diet, and effects were concurrent with increased mRNA levels of pro-inflammatory cytokines (Gao et al., 2014). Among individuals at risk for CVD, high plasma levels of TMAO is correlated to increased atherosclerotic plaque burden (Wang et al., 2011) and risk of major adverse cardiovascular events (Tang et al., 2013), and people at risk for diabetes have higher plasma TMAO concentrations (Tang et al., 2013; Lever et al., 2014; Barton et al., 2015). TMAO reductase is an enzyme that catalyzes the reduction of TMAO to trimethylamine. Our findings suggest the hypothesis that individuals with schizophrenia may have less ability to clear TMAO due to lower levels of TMAO reductase, leading to increased levels of the metabolite. In patients with schizophrenia, higher ratios of TMAO reductase were correlated with higher Framingham Risk Scores. Further blood-based metabolomics are needed to examine this hypothesis and determine levels of compounds specific to TMAO pathways present in circulation, which might provide greater insights into the role of this potential mechanism in cardiovascular disease in schizophrenia.

Kdo_2-lipid A is an essential structural component for the survival of most Gram-negative bacteria and is the active component of lipopolysaccharide (LPS), which stimulates host immune responses through a protein complex of Toll-like-receptor 4 (TLR4) and myeloid differentiation protein 2 (MD-2) (Wang et al., 2015; Raetz et al., 2006). The TLR4/MD-2 complex activates a cascade of signal transductions that orchestrate an inflammatory response. Many bacteria can modify the structure of their Kdo_2-lipid A, as a way to adapt to different environments and modulate their virulence or infectivity. Our results showing alterations in the Kdo_2-lipid A biosynthesis pathway in schizophrenia and its negative relationship with anti-inflammatory cytokine IL-10 is consistent with evidence that schizophrenia is associated with a lasting pro-inflammatory state (Kirkpatrick and Miller, 2013). To our knowledge, the clinical impact of Kdo_2-lipid A has not been established in humans; as such, more research is needed to determine how the up- or down-regulation of this pathway may impact downstream systemic inflammation. Kdo_2-lipid A has potential clinical relevance, particularly its capabilities to elicit host innate immune responses. The enzymes and receptors involved in Kdo_2-lipid A biosynthesis is an emerging target for immunopharmocological exploitation (Wang et al., 2015).

4.1. Limitations

The most important limitation of this study is its relatively small sample size, given the complex nature of schizophrenia and heterogeneity of the disorder. Additionally, only one microbiome sample was evaluated per individual. The composition of the gut microbiome is known to fluctuate across time and as a result of many factors (McDonald et al., 2018; Yatsunenko et al., 2012), and intra-individual variability may be greater than between-group variability (Flores et al., 2014). The cross-sectional design limits our ability to make causal inferences. Future prospective longitudinal studies are needed to characterize and control for temporal variations within and between groups in order to understand causal relationships between the microbiome and health in schizophrenia. This study was not designed to assess the impact of antipsychotic medications on the gut microbiome. Our clinical population of interest is individuals with long-time, chronic disease. As such, a majority of our sample were taking antipsychotic medications. Relatedly, we did not exclude individuals with schizophrenia with cardiometabolic disorders (e.g., diabetes, hypertension, heart disease). Although these conditions may influence the stability of the gut microbiota, patients with schizophrenia tend to have higher rates of these conditions; (Hennekens et al., 2005) as such, these are important to consider in understanding the phenotype of accelerated aging in schizophrenia, and excluding them would create a sample that is unrepresentative of the general schizophrenia population. It is possible that differences in the gut microbiome between groups may have been driven by these comorbid medical illnesses; however, we did not find any differences in the beta-diversity of microbial taxa ($ps > 0.38$) or functional potential ($ps > 0.20$) between persons with schizophrenia with and without these conditions, suggesting that these medical conditions were unlikely to be driving the reported findings. Likewise, we did not match samples on or control for smoking prevalence in analyses. Patients with chronic schizophrenia are markedly prone to smoke tobacco and it has been suggested that biological factors may underlie the association between this disorder and tobacco use. To disentangle the effects of smoking and diagnostic group, we compared smokers to non-smokers within the schizophrenia group did not find differences in beta-diversity of taxa ($ps > 0.068$) or functional pathways ($ps > 0.105$), suggesting that differences in microbial community composition between study groups were driven by disease rather than cigarette smoking. Functional data from PICRUSt was predicted based on 16S rRNA marker gene sequences, which does not provide direct information about the functional composition of sampled communities. Nevertheless, PICRUSt predictions have overall high accuracy (Douglas

ARTICLE IN PRESS

et al., 2019) and can yield new biological insights, particularly in a novel field of research. Future studies should incorporate shotgun metagenomic sequencing, which directly measures genetic functional potential, to validate predictions of functional potential. Finally, as this was an exploratory study, we did not have circulating levels of metabolites to further validate TMAO reductase findings. Performing blood-based metabolomics will be an important next step to discover whether compounds specific to TMAO pathways are observed in circulation, which could impact host metabolism and health.

4.2. Conclusions and next steps

Strengths of the present study include a matching of NC participants to control for other non-disease related factors known to have major influences on the gut microbiome. We build upon our previous article by utilizing a new compositionally-aware method for differential abundance testing that can be used in future studies with larger sample sizes and/or *meta*-analysis of existing datasets to help resolve inconsistencies in the extant literature. Our study is one of the few to have examined the functional potential of the gut microbiome in patients with chronic schizophrenia. Previous studies have found altered functional potentials related to metabolism of amino acids, lipids, and carbohydrates and degradation of xenobiotics (Shen et al., 2018). To our knowledge, this study is the first to show that individuals with schizophrenia have altered metabolic pathways related to TMAO reductase and Kdo_2-lipid A biosynthesis. The microbiota may impact that pathophysiology of the disease through modulation of functional pathways related to immune signaling/response and lipid and glucose regulation, which might have implications for accelerated biological aging in schizophrenia. For example, therapies that target TMAO pathways are being explored (Zeisel and Warrier, 2017), including dietary interventions targeted towards reducing TMAO levels (Leal-Witt et al., 2018; Tripolt et al., 2015).

Research is increasingly moving toward characterizing the functional capacity of the community by quantifying the abundances of genes or pathways, which can better elucidate potential downstream effects of taxonomic shifts (i.e., significant differences in abundance observed between case and control samples). Greater effort should be made to link these two facets of the microbiome (Manor and Borenstein, 2017), and future studies should focus on identifying taxonomic drivers of disease-associated functional imbalances. Deep functional profiling of the microbiome, along with integration of other omics data such as metabolomics and proteomics, offers greater promise for therapeutic discovery and finding microbiome-related interventions (Segal, 2020).

Funding sources

This work was supported, in part, by the National Institute of Mental Health (grant numbers K23 MH118435-01A1 to TTN, and 2R01 MH094151-06 and 5 T32 MH019934-24 to DVJ), the Polish National Agency for Academic Exchange (grant PPN/PPO/2018/1/00014 to TK), UC San Diego Sam and Rose Stein Institute for Research on Aging, and by IBM Research through the AI Horizons Network, UC San Diego AI for Healthy Living program in partnership with the UC San Diego Center for Microbiome Innovation.

Availability of data

Data sets generated in the current study are available in the European Nucleotide Archive of the European Bioinformatics Institute (EBI-ENA) under accession number EBI: ERP107975. Feature tables can be found in Qiita (qiita.ucsd.edu) as study ID 11,710 (prep ID 8769). All processing Jupyter Notebooks are available on GitHub at: https://github.com/knightlab-analyses/stein-schizophrenia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.10.003.

References

Casey, D.E., Hansen, T.E., Meyer, J., Nasrallah, H., 2009. Excessive mortality and morbidity associated with schizophrenia. Medical Illness and Schizophrenia. 17, 36.
Hennekens, C.H., Hennekens, A.R., Hollar, D., Casey, D.E., 2005. Schizophrenia and increased risks of cardiovascular disease. Am. Heart J. 150 (6), 1115–1121.
Lee, E.E., Hong, S., Martin, A.S., Eyler, L.T., Jeste, D.V., 2017. Inflammation in schizophrenia: cytokine levels and their relationships to demographic and clinical variables. Am. J. Geriatr Psychiatry. 25 (1), 50–61.
Kirkpatrick, B., Miller, B.J., 2013. Inflammation and schizophrenia. Schizophr. Bull. 39 (6), 1174–1179.
Kirkpatrick, B., Messias, E., Harvey, P.D., Fernandez-Egea, E., Bowie, C.R., 2008. Is schizophrenia a syndrome of accelerated aging? Schizophr. Bull. 34 (6), 1024–1032.
Nguyen, T.T., Eyler, L.T., Jeste, D.V., 2018. Systemic Biomarkers of Accelerated Aging in Schizophrenia: A Critical Review and Future Directions. Schizophr. Bull. 44 (2), 398–408.
Jeste, D.V., Wolkowitz, O.M., Palmer, B.W., 2011. Divergent trajectories of physical, cognitive, and psychosocial aging in schizophrenia. Schizophr. Bull. 37 (3), 451–455.
Clemente, J.C., Ursell, L.K., Parfrey, L.W., Knight, R., 2012. The impact of the gut microbiota on human health: an integrative view. Cell 148 (6), 1258–1270.
Carroll, I.M., Threadgill, D.W., Threadgill, D.S., 2009. The gastrointestinal microbiome: a malleable, third genome of mammals. Mamm. Genome 20 (7), 395–403.
Severance, E.G., Gressitt, K.L., Stallings, C.R., et al., 2013. Discordant patterns of bacterial translocation markers and implications for innate immune imbalances in schizophrenia. Schizophr. Res. 148 (1–3), 130–137.
Hsiao, E.Y., McBride, S.W., Hsien, S., et al., 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell 155 (7), 1451–1463.
Nguyen, T.T., Kosciolek, T., Eyler, L.T., Knight, R., Jeste, D.V., 2018. Overview and systematic review of studies of microbiome in schizophrenia and bipolar disorder. J. Psychiatr. Res. 99, 50–61.
Nguyen TT, Hathaway H, Kosciolek T, Knight R, Jeste DV. Gut microbiome in serious mental illnesses: A systematic review and critical evaluation. Schizophr Res. Published online September 5, 2019. doi:10.1016/j.schres.2019.08.026.
Nguyen, T.T., Kosciolek, T., Maldonado, Y., et al., 2019. Differences in gut microbiome composition between persons with chronic schizophrenia and healthy comparison subjects. Schizophr. Res. 204, 23–29.
Morton, J.T., Marotz, C., Washburne, A., et al., 2019. Establishing microbial composition measurement standards with reference frames. Nat. Commun. 10 (1), 2719.
Langille, M.G.I., Zaneveld, J., Caporaso, J.G., et al., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31 (9), 814–821.
Douglas GM, Maffei VJ, Zaneveld J, et al. PICRUSt2: An improved and extensible approach for metagenome inference. bioRxiv. Published online June 15, 2019:672295. doi:10.1101/672295.
Shen Y, Xu J, Li Z, et al. Analysis of gut microbiota diversity and auxiliary diagnosis as a biomarker in patients with schizophrenia: A cross-sectional study. Schizophr Res. Published online January 15, 20doi:10.1016/j.schres.2018.01.002.
Zheng, P., Zeng, B., Liu, M., et al., 2019. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. Sci. Adv.
First, M.B., Spitzer, R.L., Gibbon, M., Williams, J.B.W., 2002. Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Patient Edition With Psychotic Screen (SCID-I/PW/PSY SCREEN). Biometrics Research, New York State Psychiatric Institute.
Sheehan, D.V., Lecrubier, Y., Sheehan, K.H., et al. The Mini-International Neuropsychiatric Interview (MINI): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. J Clin Psychiatry. Published online 1998. https://psycnet.apa.org/record/1998-03251-004.
Lozupone, C.A., Stombaugh, J., Gonzalez, A., et al., 2013. Meta-analyses of studies of the human microbiota. Genome Res. 23 (10), 1704–1714.
Walters, W.A., Xu, Z., Knight, R., 2014. Meta-analyses of human gut microbes associated with obesity and IBD. FEBS Lett. 588 (22), 4223–4233.
McDonald, D., Hyde, E., Debelius, J.W., et al., 2018. American Gut: an Open Platform for Citizen Science Microbiome Research. *mSystems*. 3 (3). https://doi.org/10.1128/mSystems.00031-18.
OrganizationOthers., W.H., 2017. Guidelines for ATC Classification and DDD assignment 2018. World Health Organization. Published online, Oslo, Noruega.
Andreasen, N.C., 1983. Scale for the assessment of negative symptoms (SANS) University of Iowa. Published online, Iowa City.

AndreasenOthers., N.C., 1984. Scale for the Assessment of Positive Symptoms (SAPS). University of Iowa Iowa City.
Kroenke, K., Spitzer, R.L., Williams, J.B.W., 2001. The PHQ-9: validity of a brief depression severity measure. J. Gen. Intern. Med. 16 (9), 606–613.
Ware Jr, J.E., Sherbourne, C.D., 1992. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med. Care 30 (6), 473–483.
Parmelee, P.A., Thuras, P.D., Katz, I.R., Lawton, M.P., 1995. Validation of the Cumulative Illness Rating Scale in a geriatric residential population. J. Am. Geriatr. Soc. 43 (2), 130–137.
Wilson, P.W., D'Agostino, R.B., Levy, D., Belanger, A.M., Silbershatz, H., Kannel, W.B., 1998. Prediction of coronary heart disease using risk factor categories. Circulation 97 (18), 1837–1847.
Fernandes, B.S., Steiner, J., Bernstein, H.-G., et al., 2016. C-reactive protein is increased in schizophrenia but is not altered by antipsychotics: meta-analysis and implications. Mol. Psychiatry 21 (4), 554–564.
Frydecka, D., Misiak, B., Pawlak-Adamska, E., et al., 2015. Interleukin-6: the missing element of the neurocognitive deterioration in schizophrenia? The focus on genetic underpinnings, cognitive impairment and clinical manifestation. Eur. Arch. Psychiatry Clin. Neurosci. 265 (6), 449–459.
Thompson, L.R., Sanders, J.G., McDonald, D., et al., 2017. A communal catalogue reveals Earth's multiscale microbial diversity. Nature 551 (7681), 457–463.
Marotz, C., Amir, A., Humphrey, G., Gaffney, J., Gogul, G., Knight, R., 2017. DNA extraction for streamlined metagenomics of diverse environmental samples. Biotechniques 62 (6), 290–293.
Caporaso, J.G., Lauber, C.L., Walters, W.A., et al., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6 (8), 1621–1624.
Walters, W., Hyde, E.R., Berg-Lyons, D., et al., 2016. Improved Bacterial 16S rRNA Gene (V4 and V4–5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. mSystems. 1 (1). https://doi.org/10.1128/mSystems.00009-15.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7 (5), 335–336.
Bolyen, E., Rideout, J.R., Dillon, M.R., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37 (8), 852–857.
Amir, A., McDonald, D., Navas-Molina, J.A., et al., 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. mSystems. 2 (2). https://doi.org/10.1128/mSystems.00191-16.
Amir, A., McDonald, D., Navas-Molina, J.A., et al., 2017. Correcting for Microbial Blooms in Fecal Samples during Room-Temperature Shipping. mSystems. 2 (2). https://doi.org/10.1128/mSystems.00199-16.
McDonald, D., Price, M.N., Goodrich, J., et al., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6 (3), 610–618.
Janssen, S., McDonald, D., Gonzalez, A., et al., 2018. Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. mSystems. 3 (3). https://doi.org/10.1128/mSystems.00021-18.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26 (19), 2460–2461.
Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. 2012;40(Database issue):D109-D114.
Caspi, R., Billington, R., Fulcher, C.A., et al., 2018. The MetaCyc database of metabolic pathways and enzymes. Nucleic Acids Res. 46 (D1), D633–D639.
Shannon, C.E., 1948. A Mathematical Theory of Communication. Bell Syst. Tech. J. 27 (3), 379–423.
Faith, D.P., 1992. Conservation evaluation and phylogenetic diversity. Biol. Conserv. 61 (1), 1–10.
Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71 (12), 8228–8235.
Lozupone, C.A., Hamady, M., Kelley, S.T., Knight, R., 2007. Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. Appl. Environ. Microbiol. 73 (5), 1576–1585.
Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Published online, Ecol Monogr https://esajournals.onlinelibrary.wiley.com/doi/abs/10.2307/1942268.
Jaccard, P., 1912. The distribution of the flora in the alpine zone. 1. New Phytol. 11 (2), 37–50.
Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., Knight, R., 2013. EMPeror: a tool for visualizing high-throughput microbial community data. GigaScience 2 (1), 16.
Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Series B Stat Methodol. 57 (1), 289–300.
Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26 (1), 32–46.
Mandal, S., Van Treuren, W., White, R.A., Eggesbø, M., Knight, R., Peddada, S.D., 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microb Ecol Health Dis. 26, 27663.
Fedarko, M.W., Martino, C., Morton, J.T., et al. Visualizing 'omic feature rankings and log-ratios using Qurro. bioRxiv. Published online December 18, 2019:2019.12.17.880047. doi:10.1101/2019.12.17.880047.
Breiman, L., 2001. Random Forests. Mach Learn. 45 (1), 5–32.
Xu, Z.Z., Amir, A., Sanders, J., et al., 2019. Calour: an Interactive. Microbe-Centric Analysis Tool. *mSystems*. 4 (1). https://doi.org/10.1128/mSystems.00269-18.
Bokulich, N.A., Dillon, M.R., Bolyen, E., Kaehler, B.D., Huttley, G.A., Caporaso, J.G., 2018. q2-sample-classifier: machine-learning tools for microbiome classification and regression. J Open Res Softw. 3 (30). https://doi.org/10.21105/joss.00934.
Pedregosa, F., Varoquaux, G., Gramfort, A., et al., 2011. Scikit-learn: Machine Learning in Python. J Mach Learn Res. 12 (Oct), 2825–2830.
Virtanen, P., Gommers, R., Oliphant, T.E., et al., 2020. SciPy 1.0: fundamental algorithms for scientific computing in Python. Nat. Methods 17 (3), 261–272.
Carrero Juan Jesus, Andersson Franko Mikael, Obergfell Achim, Gabrielsen Anders, Jernberg Tomas. hsCRP Level and the Risk of Death or Recurrent Cardiovascular Events in Patients With Myocardial Infarction: a Healthcare-Based Study. J Am Heart Assoc. 2019;8(11):e012638.
Zeisel, S.H., Warrier, M., 2017. Trimethylamine N-Oxide, the Microbiome, and Heart and Kidney Disease. Annu. Rev. Nutr. 37, 157–181.
Wang, X., Quinn, P.J., Yan, A., 2015. Kdo2 -lipid A: structural diversity and impact on immunopharmacology. Biol. Rev. Camb. Philos. Soc. 90 (2), 408–427.
Zhu, F., Ju, Y., Wang, W., et al., 2020. Metagenome-wide association of gut microbiome features for schizophrenia. Nat. Commun. 11 (1), 1612.
Yatsunenko, T., Rey, F.E., Manary, M.J., et al., 2012. Human gut microbiome viewed across age and geography. Nature 486 (7402), 222–227.
Coello, K., Hansen, T.H., Sørensen, N., et al., 2019. Gut microbiota composition in patients with newly diagnosed bipolar disorder and their unaffected first-degree relatives. Brain Behav. Immun. 75, 112–118.
Painold, A., Mörkl, S., Kashofer, K., et al., 2019. A step ahead: Exploring the gut microbiota in inpatients with bipolar disorder during a depressive episode. Bipolar Disord. 21 (1), 40–49.
Zheng, P., Zeng, B., Zhou, C., et al., 2016. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. Mol. Psychiatry 21 (6), 786–796.
Naseribafrouei, A., Hestad, K., Avershina, E., et al., 2014. Correlation between the human fecal microbiota and depression. Neurogastroenterol. Motil. 26 (8), 1155–1162.
Chen, J.-J., Zheng, P., Liu, Y.-Y., et al., 2018. Sex differences in gut microbiota in patients with major depressive disorder. Neuropsychiatr. Dis. Treat. 14, 647–655.
Huang, Y., Shi, X., Li, Z., et al., 2018. Possible association of Firmicutes in the gut microbiota of patients with major depressive disorder. Neuropsychiatr. Dis. Treat. 14, 3329–3337.
Ma, X., Asif, H., Dai, L., et al., 2020. Alteration of the gut microbiome in first-episode drug-naïve and chronic medicated schizophrenia correlate with regional brain volumes. J. Psychiatr. Res. 123, 136–144.
Jiang, H., Ling, Z., Zhang, Y., et al., 2015. Altered fecal microbiota composition in patients with major depressive disorder. Brain Behav. Immun. 48, 186–194.
Sanada, K., Nakajima, S., Kurokawa, S., et al., 2020. Gut microbiota and major depressive disorder: A systematic review and meta-analysis. J. Affect. Disord. 266, 1–13.
Schloss, P.D., 2018. Identifying and Overcoming Threats to Reproducibility. Replicability, Robustness, and Generalizability in Microbiome Research. *MBio*. 9 (3). https://doi.org/10.1128/mBio.00525-18.
Forslund, K., Hildebrand, F., Nielsen, T., et al., 2015. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature 528 (7581), 262–266.
Debelius, J., Song, S.J., Vazquez-Baeza, Y., Xu, Z.Z., Gonzalez, A., Knight, R., 2016. Tiny microbes, enormous impacts: what matters in gut microbiome studies? Genome Biol. 17 (1), 217.
Aitchison, J., 1986. The Analysis of Compositional Data. Published online, London. Chapman and.
Pawlowsky-Glahn, V., Egozcue, J.J., Tolosana-Delgado, R., 2015. Modeling and Analysis of Compositional Data. John Wiley & Sons.
Knight, R., Vrbanac, A., Taylor, B.C., et al., 2018. Best practices for analysing microbiomes. Nat. Rev. Microbiol. 16 (7), 410–422.
Biddle, A., Stewart, L., Blanchard, J., Leschine, S., 2013. Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. Diversity. 5 (3), 627–640.
Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol. Lett. 217 (2), 133–139.
Flowers, S.A., Evans, S.J., Ward, K.M., McInnis, M.G., Ellingrod, V.L., 2017. Interaction Between Atypical Antipsychotics and the Gut Microbiome in a Bipolar Disease Cohort. Pharmacotherapy. 37 (3), 261–267.
Vindegaard N, Speyer H, Nordentoft M, Rasmussen S, Benros ME. Gut microbial changes of patients with psychotic and affective disorders: A systematic review. Schizophr Res. Published online January 14, 2020. doi:10.1016/j.schres.2019.12.014.
Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., Egozcue, J.J., 2017. Microbiome Datasets Are Compositional: And This Is Not Optional. Front. Microbiol. 8, 2224.
Warrier, M., Shih, D.M., Burrows, A.C., et al., 2015. The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance. Cell Rep. 10 (3), 326–338.
Wang, Z., Klipfell, E., Bennett, B.J., et al., 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472 (7341), 57–63.
Gao, X., Liu, X., Xu, J., Xue, C., Xue, Y., Wang, Y., 2014. Dietary trimethylamine N-oxide exacerbates impaired glucose tolerance in mice fed a high fat diet. J. Biosci. Bioeng. 118 (4), 476–481.
Tang, W.H.W., Wang, Z., Levison, B.S., et al., 2013. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N. Engl. J. Med. 368 (17), 1575–1584.
Lever, M., George, P.M., Slow, S., et al., 2014. Betaine and Trimethylamine-N-Oxide as Predictors of Cardiovascular Outcomes Show Different Patterns in Diabetes Mellitus: An Observational Study. PLoS ONE 9 (12), e114969.
Barton, S., Navarro, S.L., Buas, M.F., et al., 2015. Targeted plasma metabolome response to variations in dietary glycemic load in a randomized, controlled, crossover feeding trial in healthy adults. Food Funct. 6 (9), 2949–2956.
Raetz, C.R.H., Garrett, T.A., Reynolds, C.M., et al., 2006. Kdo2-Lipid A of Escherichia coli, a defined endotoxin that activates macrophages via TLR-4. J. Lipid Res. 47 (5),

ARTICLE IN PRESS

1097–1111.
Flores, G.E., Caporaso, J.G., Henley, J.B., et al., 2014. Temporal variability is a personalized feature of the human microbiome. Genome Biol. 15 (12), 531.
Leal-Witt, M.J., Llobet, M., Samino, S., et al., 2018. Lifestyle Intervention Decreases Urine Trimethylamine N -Oxide Levels in Prepubertal Children with Obesity. Obesity. 26 (10), 1603–1610.
Tripolt, N.J., Leber, B., Triebl, A., Köfeler, H., Stadlbauer, V., Sourij, H., 2015. Effect of Lactobacillus casei Shirota supplementation on trimethylamine-N-oxide levels in patients with metabolic syndrome: An open-label, randomized study. Atherosclerosis. 242 (1), 141–144.
Manor, O., Borenstein, E., 2017. Systematic Characterization and Analysis of the Taxonomic Drivers of Functional Shifts in the Human Microbiome. Cell Host Microbe 21 (2), 254–267.
Segal, E., 2020. Rich data sets could end costly drug discovery. Nature 577 (7792), S19.