OPEN

A Microbial Signature of Psychological Distress in Irritable Bowel Syndrome

Johannes Peter, MSc, Camille Fournier, MD, Marija Durdevic, MSc, Lukas Knoblich, MD, Bettina Keip, MSc, Clemens Dejaco, MD, Michael Trauner, MD, and Gabriele Moser, MD

ABSTRACT

Objective: Irritable bowel syndrome (IBS) is associated with alterations along the brain-gut-microbiota axis. Previous studies have suggested a parallel segregation of microbial features and psychological burden in IBS. This study aimed at exploring the microbial correlates of psychological distress in patients with IBS.

Methods: Forty-eight patients with IBS (Rome III criteria, M (SD) age = 42 (15) years, 35 female, 25 diarrhea-dominant, 5 constipation-dominant, and 18 alternating-type IBS) were assessed for psychological and clinical variables with validated questionnaires, fecal samples underwent microbial 16S rRNA analyses (regions V1–2). Microbial analyses comprised examination of alpha and beta diversity, correlational analyses of bacterial abundance and comparisons among subgroups defined by thresholds of psychological and IBS symptom variables, and machine learning to identify bacterial patterns corresponding with psychological distress.

Results: Thirty-one patients (65%) showed elevated psychological distress, 22 (31%) anxiety, and 10 depression (21%). Microbial beta diversity was significantly associated with distress and depression (q = .036 each, q values are p values false discovery rate–corrected for multiple testing). Depression was negatively associated with *Lachnospiraceae* abundance (Spearman's $\rho = -0.58$, q = .018). Patients exceeding thresholds of distress, anxiety, depression, and stress perception showed significantly higher abundances of *Proteobacteria* (q = .020–.036). Patients with anxiety were characterized by elevated *Bacteroidaceae* (q = .036). A signature of 148 unclassified species accounting for 3.9% of total bacterial abundance co-varied systematically with the presence of psychological distress.

Conclusions: Psychological variables significantly segregated gut microbial features, underscoring the role of brain-gut-microbiota interaction in IBS. A microbial signature corresponding with psychological distress was identified.

Clinical Trial Registration: ClinicalTrials.gov identifier NCT02536131, retrospectively registered.

Key words: anxiety, depression, gut-brain-microbiome axis, irritable bowel syndrome, machine learning, psychological distress.

INTRODUCTION

The understanding of the dynamic interactions between brain and behavior, the gastrointestinal system, and gut microbiota is rapidly evolving (1,2). Psychobiological stress reactions inevitably lead to shifts of homeostasis along the brain-gut-microbiota axis, and the bacterial microbiome is increasingly recognized as a relevant entity within this interplay (3–5). Although coordinated physiological stress responses are necessary for survival, chronic exposure to stress may predispose to dysregulation along the brain-gut-microbiota axis, immune imbalances and to the development of stress-related disorders (6–8). A role of stress in conjunction with microbial factors is also highly presumed in the pathogenesis of irritable bowel syndrome (IBS) (9–11). The underlying interaction between stress and the microbiome seems to be bidirectional: there is evidence both for stress-induced changes in the intestinal microenvironment and the microbiome, and vice versa, effects of microbiome-related factors on psychobiological stress reactivity. Regarded from a biopsychosocial perspective, environmental factors, behavior and psychological appraisal processes hereby interact with central and autonomous nervous, endocrine, metabolic, and immune functions and the gastrointestinal microbiota via multidirectional pathways (12).

Top-Down Pathways

The release of stress hormones impacts the gut ecosystem by modulating gastrointestinal blood flow, secretion, permeability, and motility, as well as activation of the immune system (13,14). Numerous animal experiments have shown that chronic stress alters the microbiome, with general decreases in diversity and richness or shifts in composition (15–19). Microbial changes may in turn

HADS = Hospital Anxiety and Depression Scale, **IBS** = irritable bowel syndrome, **LEfSe** = linear discriminant analysis effect size, **OTU** = operational taxonomic unit (bacteria)

SDC Supplemental Content

From the Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, Austria (Peter, Fournier, Knoblich, Keip, Dejaco, Trauner, Moser); and Center for Medical Research, Medical University of Graz, Austria (Durdevic).

Address correspondence to Johannes Peter, MSc, Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Wien, Austria. E-mail: johannes.peter@meduniwien.ac.at

J.P. and C.F. share co-first authorship.

Received for publication October 13, 2017; revision received July 10, 2018.

DOI: 10.1097/PSY.0000000000000630

contribute to inflammatory states, altered neurochemistry, visceral pain, and behavioral abnormalities, altogether resembling the picture of IBS. Experimental stress paradigms have therefore been proposed as animal models of IBS (20,21).

Bottom-Up Pathways

Microbiota transplantation from anxious patients with diarrhea-predominant IBS to mice triggered heightened intestinal transit, loss of barrier function, immune activation, and anxiety-like behavior in recipient mice (22). Studies with germ-free animals have demonstrated an important role of microbiota for development and programming of the hypothalamic-pituitary-adrenal axis and behavior (23,24). These mechanisms seem to have pronounced sex-specific aspects (25,26). Also beyond developmentally sensitive temporal windows, gut microbes can affect basal circuits of emotion processing and the autonomous nervous system via ascending vagal pathways from the intestinal lumen (27,28). Interoceptive stimuli can interfere with conscious and subconscious emotional and cognitive processing (29). In this context, the stability of microbial ecosystems can be considered a determinant of gastrointestinal homeostasis with a relevance for appraisals of stress (30). Furthermore, bacterial metabolic pathways affect essential neurotransmitter systems (31,32) and a number of studies point to metabolic and epigenetic effects on the brain with impact on social cognition, reward, and emotion processing (33–35).

Initial evidence demonstrates associations with emotion processing also in humans (36,37), and gut microbial associations with brain morphology and temperamental traits have been identified (38,39). The association between psychopathology and gastrointestinal conditions is widely observed. Microbial shifts were found in depression (40,41), autism (42), as well as inflammatory and functional gastrointestinal diseases (43).

IBS is a brain-gut axis disorder (10,44) associated with high rates of psychopathology (45,46) and altered stress reactivity patterns (47–49). Further mechanisms present in IBS comprise immune activation, heightened gut permeability, alterations in tryptophan and bile metabolism, and pain processing (50–56).

Microbial Characteristics of IBS

A number of studies have attempted to characterize gut microbiomes in IBS compared with healthy controls, which is however complicated by inconsistency and large overlap with healthy microbiomes. Some key findings with a relative concordance among studies include reductions in alpha diversity and richness, depletion of *Bifidobacteria*, elevated abundance of *Proteobacteria*, and elevations in *Ruminococcus* species (10,43,57–60). A heightened *Firmicutes:Bacteroidetes* ratio has been observed repeatedly in subgroups of patients with IBS. Several recent studies have reported reductions of bacteria producing methane and/or producing short-chain fatty acids (61–64), but here again, exceptions have been observed (65). One study identified bacteria associated with IBS severity by machine learning techniques, which resulted in a pattern of taxa disseminated over the whole phylogenetic tree (63).

Several observations across studies point to associations between psychological variables and microbiota in IBS. It was repeatedly found that psychological distress segregated patients with IBS in parallel with their microbial composition (66,67) and a recent study reported associations between early life trauma and microbial features in IBS (68). Moreover, certain bacterial features (e.g., heightened *Proteobacteria* and depleted *Bifidobacteria*) were described in both depression and IBS (40,58,69), and it has been observed that the fecal microbiome of patients with IBS presents strong similarities with that of depressive patients (70).

The aim of this study was, therefore, to explore associations between gut microbial characteristics and psychological distress, anxiety, and depression in patients with IBS.

METHODS

Recruitment

The study was conducted at the University Hospital of Vienna, Outpatient Clinic for Psychosomatics at the Department of Gastroenterology and Hepatology, University Clinic of Internal Medicine III. It included patients with IBS diagnosed according to Rome III criteria, aged between 18 and 89 years, and refractory to other IBS therapies. Exclusion criteria were pregnancy, bowel surgery, mental retardation, insufficient knowledge of German, concomitant severe organic disease or schizophrenia, psychosis, substance-related disorder or panic disorder, and antibiotic treatment within the month before stool collection. Screening for eligibility was performed in routine first interviews at the outpatient clinic under consideration of the hospitals' medical records. The study was conducted between August 2014 and August 2016. Sixty-three patients with IBS were consecutively screened for eligibility, of which five patients declined to participate, five did not meet the inclusion criteria, and 53 were enrolled. Three of these did not provide complete data (questionnaires and stool), in one case microbiome data quality was insufficient, and one was excluded from the analyses because of acute inflammation at the time of sample collection. Complete data sets for analysis were ultimately available from 48 patients (see flowchart in Figure S1, Supplemental Digital Content 1, http://links.lww.com/PSYMED/A505). The study protocol was approved by the ethics committee of the Medical University of Vienna (ID: 1502/2014). Informed consent was given by each participant. No financial or other incentives were offered for study participation.

Questionnaires

Anxiety, *depression*, and *psychological distress* were assessed with the Hospital Anxiety and Depression Scale (German version, HADS), (71) a screening instrument for primarily somatically ill patients. Each of the two anxiety and depression scales has seven items, with a four-step response set (scores 0–21 each). Reported internal consistency is Cronbach's α value of .80 (72). Anxiety, depression, and psychological distress (the latter is the sum of anxiety and depression) entered the analyses as continuous scores as well as dichotomized categorizations. Dichotomization was performed for scores higher than 10 to indicate clinically relevant anxiety and depression, respectively. The dichotomous categorization *absence/presence of psychological distress* was the primary variable in this study. It was defined by HADS anxiety or depression scores higher than 10, or a combined score of 16 or higher. This criterion was developed for screening need for mental health support in patients with cancer in a large-scale study and has proven good practicability and validity (73).

Perceived stress was measured with the Perceived Stress Questionnaire, German version, (74) an instrument assessing subjectively experienced stress independent of a specific and objective occasion with 20 items and 4-step response sets. Cronbach's α is .85 or greater for the overall score of the German version (74). Scores were linear transformed to values between 0 and 1, entered the analyses as a continuous variable, and categorized for subgroup analyses based on norm values from healthy adults (mean (SD) stress perception = .33 (0.1)) (74–76). Values greater than one standard deviation above the mean (>0.50, corresponding to the upper 16.7% of the norm) of healthy adults were classified as elevated.

The IBS symptoms *abdominal pain*, *bloating*, *diarrhea*, and *constipation* were assessed by single visual analog scales (0, not at all present, to 100, extremely pronounced) as in a previous study (77).

IBS severity was assessed with the Irritable Bowel Syndrome – Severity Scoring System (78), a questionnaire for clinical assessment of IBS symptom burden and severity. Values range between 0 and 500, with higher values representing higher symptom burden. Values were classified as mild (values ranging 75–175), moderate (175–300), and severe IBS (300–500) as proposed. Sound reproducibility, sensitivity, and specificity are reported for the German version (79), and the scale has been recommended repeatedly for assessment of IBS in methodological reviews (80,81).

Microbiome Analyses

16S Ribosomal RNA Sequencing

Stool samples were collected and frozen by patients, brought to the hospital and deep frozen at −80°C. DNA isolation, library preparation, and sequencing were then performed at the Graz University Center for Medical Research as described in Klymiuk et al. (2016) (82). Frozen stool samples were used for total DNA isolation by combination of mechanical and enzymatic lysis with the MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi; Roche, Mannheim, Germany) according to manufacturer's instructions. Samples were bead beaten for mechanical lysis at 6500 rpm for 30 seconds twice in a MagNA Lyser (Roche). After incubation with lysozyme and Proteinase K, enzymes were deactivated at 95°C for 10 minutes and DNA purification was performed according to kit instructions. PCR amplification was performed with the target specific primers 27f and 357r and 2 μl of total DNA extract was used for a 25 μl of PCR reaction in triplicates containing 1 × Fast Start High Fidelity Buffer, 1.25 U High Fidelity Enzyme, 200 μM dNTPs, 0.4 μM bar-coded primers, and PCR-grade water (Roche). The final library was quantified using a Quantus Fluorometer (Promega, Mannheim, Germany) and loaded to an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using a high-sensitivity DNA assay according to manufacturer's instructions for quality control. A 6pM library run was performed on a MiSeqII desktop sequencer (Illumina, Eindhoven, the Netherlands) with 20% PhiX control DNA. The resulting FASTQ files were deposited at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA386442. MiSeq paired-end raw sequence forward and reverse reads were subsequently merged using ea-utils v1.1.2 with standard settings, followed by a split library step from QIIME v1.9.1 and removal sequence reads shorter than 200 nucleotides, reads that contained ambiguous bases, or reads with an average quality score of less than 30. Chimera were removed using USEARCH v6. against 97% clustered SILVA reference database (83). Operational taxonomic units (OTUs) were picked using the QIIME open-reference pipeline to perform clustering steps at 97% sequence similarity, the taxonomy assignment with a UCLUST algorithm, alignment of reference sequences with pyNAST, and generation of a phylogenetic tree with FastTree. An introduction to basic concepts in microbiological ecology relevant to this study is provided in Supplemental Digital Content 2, http://links.lww.com/PSYMED/A506.

Diversity and Composition Analyses

Alpha diversity was analyzed at a rarefaction depth of 20,000 sequences using the *observed species*, *Faith's phylogenetic diversity* (PD) and *estimated richness* (*Chao1*) metrics. Alpha comparisons among subgroups were performed by Mann–Whitney *U* tests and 999 Monte–Carlo permutations.

Beta-diversity analyses were performed using abundance-weighted UniFrac distances as phylogenetic estimates of community similarity. Associations with psychological, clinical, and demographic variables were tested with Adonis (vegan package 2.4-0), a permutational analysis of variance.

Subgroup and Correlation Analyses of Bacterial Abundance

Correlations between OTUs and study variables were calculated using a Spearman correlation after removing OTUs with less than 20% occupancy. Subgroup analyses of bacterial abundance were performed using Linear discriminant analysis effect size (LEfSe (84)), a method to determine bacteria most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding effect relevance. It identifies bacterial taxa differentially abundant between classes by nonparametric Kruskal-Wallis tests. Significance is subsequently investigated by pairwise Wilcoxon rank-sum tests, and effect sizes are estimated by linear discriminant analyses (85). The lower threshold for reporting logarithmic linear discriminant analyses scores was set at 2.0 (default).

Machine Learning

With regard to the interactional nature of the microbiome and to overcome the boundaries of repeated testing, modeling via machine learning was adopted to complement the analyses. Machine learning was applied after removal of OTUs with less than 10 reads per single sample and feature selection by gradient-boosting classifier (scikit-learn 0.18.1), an algorithm combining weak learner-decision trees and boosting (100 times) to optimize cost function over function space. Each individual decision tree in the gradient-boosting algorithm intrinsically performs feature selection by selecting appropriate split points. This information was used to measure the importance of each feature: the more often a feature was used in the split points of a tree the more important was the feature (weighting). The feature importance was then averaged across all of the decision trees within the model. The sample was randomly split into a training set (32 samples) and a prediction set (16 samples). A model based on the training data was built using the Random Forest Classifier (86,87) with 100 trees and parameter adjusted by GridSearchCV using three-fold cross validation.

Statistical Analyses

Statistical analyses were conducted in QIIME (88), the bioinformatics platform Galaxy (89), R (90), and SPSS 23 (SPSS Inc). Parameter-free tests were chosen throughout the analyses because assumptions of normal distribution were violated in several variables. With regard to the psychological and IBS symptom-related variables, continuous scores were used in the analyses of associations with microbial composition (Adonis tests) and correlation analyses with bacterial abundance. Categorical variables were used in subgroup analyses (alpha diversity, abundance testing with LEfSe) and for the machine learning classifier. *P* values were corrected by Bonferroni's method as default in QIIME or otherwise corrected by Benjamini and Hochberg's method to control for the false discovery rate and given as *q* values. The α level was set at .05 (two-sided) throughout all tests.

RESULTS

Sample Characteristics

Complete data sets, including questionnaires and stool samples, were available from 48 patients with IBS aged 42 (15) years, experiencing IBS for 9 (9) years, and with 35 (73%) female participants. Age ranged between 19 and 70 years and was distributed relatively even with a median of 42 years and two peaks below (at approximately 30 years) and above (at approximately 60 years). With 73% female participants, sex distribution was clearly skewed. Twenty-five patients (52%) experienced IBS with diarrhea (IBS-D), 18 (38%) from IBS with mixed symptoms (IBS-mix), and 5 (10%) from IBS with constipation (IBS-C). A postinfectious onset of IBS (PI-IBS) was known in 9 (19%) of the sample. With a M (SD) severity (Irritable Bowel Syndrome – Severity Scoring System) of 310.3 (77.5), IBS was classified as mild in 3 (6%) patients, moderate in 17 (35%) patients, and severe in 28 (58%) patients. Antidepressants were taken by 9 (19%), Mebeverine by 4 (8%), and proton pump inhibitors by 3 (6%) patients. Further medications each taken by a single patient were the following: cholestyramine,

ursodeoxycholic acid, and domperidone. Two patients reported the intake of a probiotic nutritional supplement.

Psychological Distress

As frequently found in IBS populations and particularly in patients of specialized tertiary centers, there was a high proportion of psychological burden in the sample: 23 (48%) had a psychological disorder in the past or during the study, 21 (44%) were in a psychotherapy during the study, and 38 (79%) reported impairing psychosocial conditions (financial/job strain, history of trauma, family or interpersonal problems). Anxiety was 9.6 (4.13), depression was 7.09 (3.92), and psychological distress was 16.52 (7.42) points high. According to the previously mentioned thresholds (see Methods), 31 patients had elevated psychological distress, 22 had anxiety, and 10 had depression, with 7 experiencing both anxiety and depression (Figure 1A). The distribution of sample characteristics among subgroups with or without clinically relevant psychological distress is shown in Table 1.

Perceived stress and psychological distress were highly correlated (Spearman's $\rho = .83$, $p < .001$). Perceived stress was .537 (.200) in the whole sample, and 34 (71%) patients displayed elevated stress perception. Perceived stress values showed a bimodal distribution (Figure 1B).

Interestingly, no significant correlations between psychological distress and IBS severity or IBS single symptoms (abdominal pain, diarrhea, bloating, constipation) were found; a scatter plot and exact statistics are provided in Supplemental Digital Content 3, http://links.lww.com/PSYMED/A507.

Microbial Analyses

The hypervariable regions V1 and V2 of 16S rRNA genes were sequenced from 48 fecal samples and yielded 3067522 sequences and an M (SD) number of MiSeq reads of 63907 (12077) per sample, with a range from 26283 to 84351 sequences. After filtering OTUs with less than 20 total counts and presence in less than 5 samples, the OTU table contained 6.379 OTUs.

Bacterial Diversity and Composition

The bacterial diversity in the fecal samples (alpha diversity) was compared between patients with and without psychological distress (subgroup analyses based on dichotomized categorization). Diversity in patients with psychological distress was slightly lower than in those without distress, but the difference was not significant (Figure 2, Table 2). Diversity between patients with and without elevated stress, anxiety or depression was also compared, as well as the different IBS subtypes and severity, but there were no significant differences among any of the subgroups (values are summarized in Table S4, Supplemental Digital Content 4, http://links.lww.com/PSYMED/A508).

Continuous psychological variables and measures of bacterial composition (weighted beta diversity, which takes into account the abundance of taxa and is therefore sensitive to systematic alterations in large microbial groups) were examined in subsequent analyses. Microbiome composition was significantly associated with psychological distress and depression (Table 3, Adonis testing of abundance-weighted UniFrac distances). Other tested psychological and IBS symptom-related variables showed no association, and the same was found for previously identified microbial covariates age, sex, and antidepressant intake (91–93).

To provide a higher-order perspective on the microbial communities present in the study cohort, the 48 fecal samples were clustered according to their microbiome composition (hierarchical clustering with unweighted pair group method with arithmetic mean of weighted UniFrac distances as shown in Figure 3). This resulted in a two-cluster solution with dissimilarities of 0.39 (0.07) between clusters and 0.27 (0.05) within the clusters.

Distributions of microbial and patient characteristics were tested between the two clusters. *Firmicutes* and *Bacteroidetes* are the two dominant phyla in the human gut, and the *Firmicutes* to *Bacteroidetes* (F:B) ratio thus provides a rough estimate of the composition of a microbial community. It amounted to a median (interquartile range) of 1.92 (1.47–2.51) in the 28 patients of cluster 1 and 12.21 (6.91–28.83) in the 20 patients of cluster 2. The difference was significant at $p < .001$ (Mann–Whitney U test). Microbial alpha diversity was almost equal in cluster 1 (*Chao1* = 2232 (409)) and in cluster 2 (2196 (497), $p = .76$, Mann–Whitney U test and Monte–Carlo permutation). Distributions of psychological and IBS symptom-related variables in the two microbial clusters are given in Table 4. Distress, depression, and perceived stress were higher in cluster 1, but significances collapsed

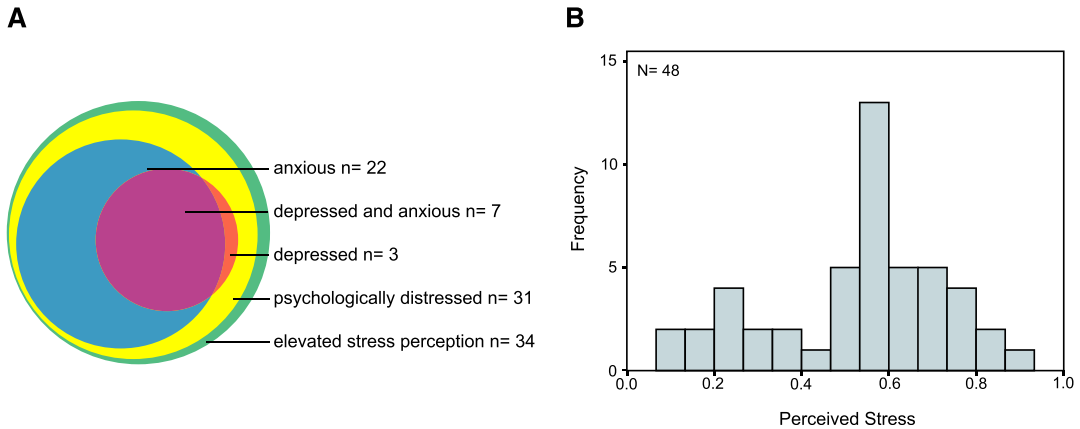

FIGURE 1. Venn diagram of different aspects of psychological burden present in the sample (A). Elevated stress perception was inclusive of psychological distress, psychological distress was inclusive of anxiety and depression, and anxiety and depression overlapped in 7 patients. Histogram of perceived stress values showed a bimodal distribution (B). Color image is available only in online version (www.psychosomaticmedicine.org).

TABLE 1. Sample Characteristics in Patients Without Versus With Psychological Distress

	Not Distressed, $n = 17$	Distressed, $n = 31$	p	q
Age, y	33.0 (25.5–54.5)	45.0 (32.0–56.0)	.19	.46
Disease duration, y	7.0 (4.5–12.0)	6.0 (3.0–10.0)	.39	.67
Sex, male/female	6/11	7/24	.34	.65
PI-IBS	3	6	.89	.93
IBS-D	9	16	.73	.82
IBS-C	1	4	.73	.82
IBS-mix	7	11	.73	.82
IBS severity	297 (259–359)	335 (266–371)	.52	.82
Abdominal pain	46.0 (34.5–62.0)	61.5 (45.3–72.0)	.082	.22
Bloating	64.0 (50.5–80.5)	64.0 (57.0–75.0)	.97	.97
Diarrhea	39.0 (4.3–54.3)	53.5 (29.3–66.0)	.054	.18
Constipation	21.0 (2.5–69.0)	20.0 (2.8–52.8)	.69	.82
Debilitating psychosocial conditions	13	25	.73	.82
Taking on psychotherapy	6	15	.382	.84
Diagnosis of psychological disorder	5	18	.057	.18
Perceived stress (PSQ)	0.325 (0.215–0.450)	0.620 (0.570–0.720)		
Psychological distress (HADS)	6.5 (10.0–11.5)	20.0 (18.0–23.0)		
Anxiety	5.0 (3.0–7.0)	12.0 (10.0–14.0)		
Depression	3.0 (2.0–6.0)	8.0 (6.0–11.0)		

IBS = irritable bowel syndrome; PSQ = Perceived Stress Questionnaire; HADS = Hospital Anxiety and Depression Scale.

Median (interquartile range) are shown for metric data, p values are two-sided from Mann–Whitney U tests, and χ^2 tests for balance of nominal data. Q values are p values corrected for multiple comparisons by Benjamini and Hochberg's method. Higher values mean higher symptom burden for all questionnaire data.

after false discovery rate correction. IBS symptom-related aspects, such as symptom severity and subtype, and postinfectious onset of IBS were evenly represented in both clusters.

Correlational Analyses

Correlational analyses between relative abundance (species and higher taxonomic levels) and psychological or IBS symptom-related variables (continuous scores) yielded 11 associations remaining significant after controlling for multiple testing, all with *Firmicutes* members. Anxiety was correlated with the genus *Anaerotruncus* (Spearman's $\rho = .65$, $q = .001$), depression with the family *Lachnospiraceae* ($\rho = -.58$, $q = .018$), as well as seven unclassified species given in Table S5 (Supplemental Digital Content 5, http://links.lww.com/PSYMED/A509). IBS severity correlated with the

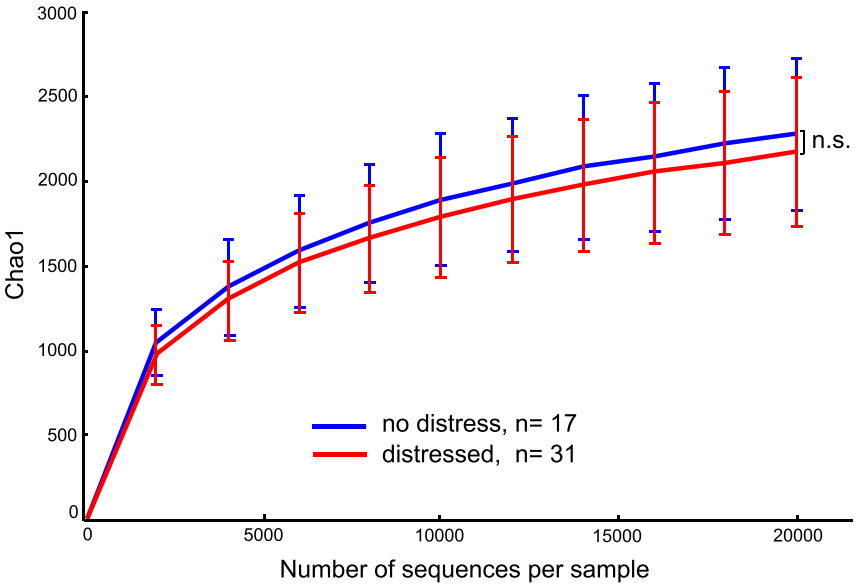

FIGURE 2. Alpha diversity rarefaction curves of estimated bacterial richness (*Chao1*) of patients with versus without psychological distress (HADS). Color image is available only in online version (www.psychosomaticmedicine.org).

TABLE 2. Alpha Diversity in Patients With Versus Without Psychological Distress

	Subgroups Split by Presence of Psychological Distress			
	No Distress, $n = 17$	Distressed, $n = 31$	p	q
Observed species	1539 (277)	1443 (251)	.24	.69
Phylogenetic diversity	5046 (795)	4951 (749)	.69	.69
Chao1	2285 (455)	2179 (439)	.46	.69

Observed species, Phylogenetic Diversity, and *Chao1* are metrics depicting different aspects of alpha diversity with M (SD) given. *P* values from Mann–Whitney *U* tests and Monte–Carlo permutation, *q* values (*p* values corrected for multiple comparisons by Benjamini and Hochberg's method).

genera *Ruminococcus* ($\rho = .62$, $q = .005$) and *Coprococcus* ($\rho = .59$, $q = .013$).

Bacterial Abundances Between Subgroups Defined by Psychological Variables

The *Firmicutes* to *Bacteroidetes* ratio was lower, albeit non-significantly, in patients with psychological distress (median (interquartile range) = 2.25 (1.84–5.22) compared with those without psychological distress (median (interquartile range) = 6.56 (2.02–13.66), $p = .091$, Mann–Whitney *U* test. Analyses of relative bacterial abundance between subgroups were performed using LEfSe, a method for detection of significantly elevated bacterial taxa. The presence of psychological distress was associated with four elevated *Proteobacteria* members (phylum to family level) and one *Bacteroidetes* genus (*Barnesiella*). Two *Bacteroidetes* members and the same four *Proteobacteria* members as in psychological distress were increased in elevated perceived stress. Anxiety was associated with five *Bacteroidetes* members and the *Proteobacteria* phylum. The heightened occurrence of *Bacteroides* (genus) and *Bacteroidaceae* (family) in anxious patients showed the highest effect magnitudes among the comparisons by psychological variables. *Proteobacteria* phylum and the *Prevotellaceae* family were elevated in depression. Elevated bacterial taxa from subgroups defined by psychological variables are given in Table 5; all differentially abundant bacteria identified in subgroup analyses are marked in Figure 4.

Bacterial Abundances Between Subgroups Defined by IBS Symptom Variables

Likewise, patient subgroups by IBS symptom-related variables were tested for differences in bacterial abundance. Because the mild IBS group contained only three patients, the mild and moderate IBS groups were combined and compared with severe IBS. The LEfSe analysis yielded three *Bacteroidetes* members (significant from phylum to order level), and three *Firmicutes* members associated with mild/moderate IBS. Postinfectious onset history of IBS was associated with an unclassified *Erysipelotrichaceae* genus. Patients with diarrhea-predominant IBS had higher *Pseudobutyrivibrio* genus, whereas IBS-mix patients had three elevated *Propionibacterium* taxa (members of *Actinobacteria* phylum, significant from order to genus level) (Figure 4, Table 6).

Machine-Learning Model

Machine learning permits modeling of complex and interactional bacterial signatures, and their use in microbiome research has been encouraged by expert panels. We therefore attempted to complement our analyses by a machine learning approach. A boosting algorithm selected 148 bacteria (altogether a "bacterial signature") from the whole data set of 48 samples corresponding with presence of psychological distress. With a count of 122.163 sequences, these bacteria covered approximately 3.9% of the total bacterial abundance. The data set was then split in a training ($n = 32$) and a test set ($n = 16$). A Random Forest model was trained to predict the presence or absence of psychological distress in the training set from the relative abundances of the signature bacteria. The bacteria were ranked according to their prediction feature importance (Figure 5; see Methods for detailed information on machine learning and feature ranking). The Random Forest model's prediction accuracy was subsequently demonstrated in the test set ($n = 16$). With an area under the receiver operating characteristic curve of 0.98, the model showed a high prediction accuracy (see Figure S6, Supplemental Digital Content 6, http://links.lww.com/PSYMED/A510). However, the conclusiveness of this analysis is tainted by the fact that feature selection was performed on the whole data set including the test set. This was necessary because of the small sample size, as machine learning feature selection techniques require at least ~50 data sets, but is associated with a high likelihood of overfitting (94).

The complete list of signature bacteria, their taxonomic classification, model importance, and representative sequences are given in Table S7 (Supplemental Digital Content 7, http://links.lww.com/PSYMED/A511). The top 20 features were all unclassified species, as was confirmed by blast analyses of representative sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Twelve of the top 20 features were members of the *Lachnospiraceae* family, and three were members of the *Ruminococcaceae* family (both members of *Clostridiales* order). These two families were also dominant in the whole set of 148 species, where taken together, they represented

TABLE 3. Associations Between Weighted Composition, Psychological Distress, Anxiety, and Depression

	Pseudo-F	R^2	p	q
Psychological variables				
Distress (HADS)	2.97	0.061	.008**	.036*
Anxiety	2.12	0.044	.029*	.087
Depression	3.09	0.063	.004**	.036*
Perceived stress (PSQ)	1.46	0.031	.13	.24
IBS symptom-related variables				
IBS severity (IBS-SSS)	0.69	0.015	.73	.77
IBS subtype	0.65	0.014	.77	.77
Potential covariates				
Sex	0.83	0.018	.56	.72
Age	0.86	0.018	.53	.72
Antidepressant intake	14.84	0.031	.13	.24

HADS = Hospital Anxiety and Depression Scale; IBS = irritable bowel syndrome; PSQ = Perceived Stress Questionnaire.

Pseudo-F, coefficient of determination R^2, *p*, and *q* values (*p* values corrected for multiple comparisons by Benjamini and Hochberg's method) from Adonis tests of weighed UniFrac distances.

* $p < .05$.

** $p < .01$.

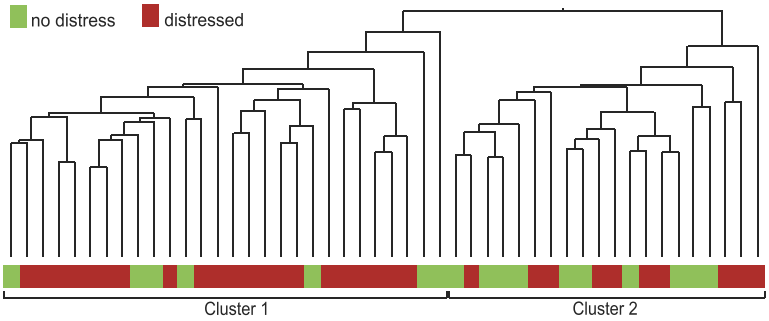

FIGURE 3. Hierarchical dendrogram based on weighted UniFrac distance. Unweighted Pair Group Method with Arithmetic mean clustering revealed 2 clusters with 28 patients in cluster 1 and 20 patients in cluster 2. Patients with psychological distress are indicated in red (dark gray in print grayscale image), those without psychological distress in green (light grey in print). Color image is available only in online version (www.psychosomaticmedicine.org).

66% of the signature OTUs. At the phylum level, most OTUs were members of *Firmicutes* (120 OTUs), followed by *Bacteroidetes* (20 OTUs), whereas *Actinobacteria* counted two OTUs, and *Proteobacteria* one, and five OTUs were unclassified up to phylum level (belonging to unknown bacteria).

DISCUSSION

This study assessed associations between psychological factors and gut microbiota in IBS by using several methods of microbial analysis, including tests of diversity and composition, correlational analyses, subgroup comparisons, and machine learning techniques.

Limitations

Substantial limitations of this study are the lack of a control group and the small sample size. The results must therefore be interpreted with caution, and further studies are required to determine associations between psychological factors and gut microbiota in patients with IBS, and importantly, also in healthy individuals.

Furthermore, the outcomes of this study were highly dependent on self-report assessments with the HADS. The HADS has been subjected to criticism because of psychometric shortcomings (95), concerning the validity of the subscales anxiety and depression. Our results regarding anxiety and depression should therefore be interpreted carefully. A strength of the HADS however is its familiarity to many clinicians and its wide use in the field (63,66,68), which allows for comparisons across studies.

Another weakness can be seen in the specific OTU clustering technique used in this study, which is implemented in QIIME (88) and has been widely used but was found to inflate OTU counts under certain circumstances (96,97). Methodological advances have recently been made with regard to sequence clustering, and a possible transition from OTUs to higher resolution amplicon sequence variants has been proposed (98–100).

Microbial analyses are also afflicted with general limitations, for example, loss of information in beta-diversity analyses, or possibly misleading results by combination and analysis of bacteria at higher phylogenetic levels without sufficient resolution, the latter also related to the fact that many species are currently not even named. A detailed understanding of microbiome-body interactions cannot be reached until in-depth characterization of these unclassified bacteria has been achieved (e.g., their role in bile acid metabolism or production of neurotransmitter precursors (31,101)).

TABLE 4. Distribution of Main Study Variables Among Microbial Clusters

	Cluster 1, $n = 28$	Cluster 2, $n = 20$	p	q
Psychological variables				
Distress (HADS)	19.5 (15.0–23.0)	15.5 (9.3–18.0)	.016	.056
Anxiety	11.0 (7.0–14.0)	9.0 (6.3–11.8)	.063	.11
Depression	8.0 (5.0–11.0)	6.0 (2.3–7.0)	.013*	.056
Perceived stress (PSQ)	0.60 (0.55–0.69)	0.53 (0.27–0.59)	.044*	.10
IBS variables				
Symptom severity	319 (257–375)	316 (282–367)	.98	.98
PI-IBS	5 (18%)	4 (20%)	.85	.98
Subtype IBS-D	13 (46%)	12 (60%)	.49	.68
Subtype IBS-mix	11 (39%)	7 (35%)	.49	.68
Subtype IBS-C	4 (14%)	1 (5%)	.49	.68

HADS = Hospital Anxiety and Depression Scale; IBS = irritable bowel syndrome; PSQ = Perceived Stress Questionnaire.

p values are two-sided from Mann–Whitney U tests, and χ^2 tests for the balance of nominal data. Data shown as median (interquartile range) or cases (percent). q values are p values corrected for multiple comparisons by Benjamini and Hochberg's method.

* $p < .05$.

TABLE 5. Elevated Bacteria in Patient Subgroups Defined by Psychological Variables

Letter in Figure 5	Name	Taxonomic Level	LDA	*p*	*q*
Presence of psychological distress					
l	*Proteobacteria*	Phylum	2.51	.008**	.021*
n	*Burkholderiales*	Order	2.27	.007**	.020*
m	*Betaproteobacteria*	Class	2.27	.007**	.020*
o	*Alcaligenaceae*	Family	2.14	.005**	.020*
f	*Barnesiella*	Genus	2.02	.022*	.036*
Elevated stress perception					
d	*Alistipes*	Genus	2.78	.023*	.036*
c	*Rikenellaceae*	Family	2.78	.024*	.036*
l	*Proteobacteria*	Phylum	2.50	.007**	.020*
n	*Burkholderiales*	Order	2.20	.007**	.020*
m	*Betaproteobacteria*	Class	2.20	.007**	.020*
o	*Alcaligenaceae*	Genus	2.13	.009**	.021*
Anxiety					
h	*Bacteroidaceae*	Family	3.01	.022*	.036*
g	*Bacteriodes*	Genus	3.01	.022*	.036*
d	*Alistipes*	Genus	2.77	.043*	.043*
c	*Rikenellaceae*	Family	2.77	.043*	.043*
l	*Proteobacteria*	Phylum	2.47	.025*	.036*
f	*Barnesiella*	Genus	2.15	.030*	.040*
Depression					
e	*Prevotellaceae*	Family	2.92	.028*	.038*
l	*Proteobacteria*	Phylum	2.16	.020*	.036*

Bacteria with significantly elevated abundance in the respective category/subgroup as identified in LEfSe analyses.

p and *q* values and LDA effect sizes from LEfSe analyses.

* $p < .05$.

** $p < .01$.

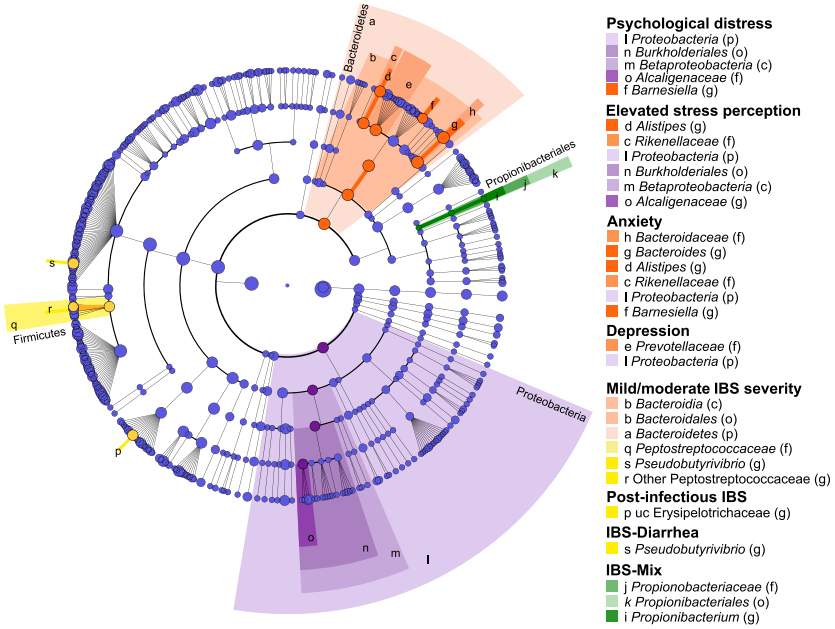

FIGURE 4. Cladogram from LEfSe analyses with differentially abundant taxa. Marked taxa display significant higher relative abundance in patients belonging to the named category. uc = unclassified. Phylogenetic levels are indicated by letters in brackets (*p* = phylum, c = class, o = order, f = family, g = genus). See Tables 5 and 6 for significances and effect sizes. Color image is available only in online version (www.psychosomaticmedicine.org).

TABLE 6. Elevated Bacteria in Patient Subgroups Defined by IBS Symptom Variables

			LDA	*p*	*q*
Mild/Moderate IBS					
b	*Bacteroidia*	Class	3.25	.038*	.043*
b	*Bacteroidales*	Order	3.25	.038*	.043*
a	*Bacteroidetes*	Phylum	3.25	.038*	.043*
q	*Peptostreptococcaceae*	Family	2.43	.007**	.020*
s	*Pseudobutyrivibrio*	Genus	2.36	.003**	.020*
r	Other Peptostreptococcaceae	Genus	2.10	.040*	.043*
Postinfectious IBS					
p	uc Erysipelotrichaceae	Genus	2.74	.043*	.043*
IBS-diarrhea					
s	*Pseudobutyrivibrio*	Genus	2.73	.019*	.036*
IBS-mixed					
j	*Propionibacteriaceae*	Family	2.38	.002**	.020*
k	*Propionibacteriales*	Order	2.19	.002**	.020*
i	*Propionibacterium*	Genus	2.11	.002**	.020*

IBS = irritable bowel syndrome; uc = unclassified bacterium.

Bacteria with significantly elevated abundance in the respective category/subgroup as identified in LEfSe analyses.

* $p < .05$

** $p < .01$.

Furthermore, it has been argued that functional properties can differ significantly even below the 97% genetic similarity criterion (102). In general, observing alterations of single bacteria can be misleading, because it remains unclear to which extent they are the results of microbe-microbe or microbe-host interactions. Taking into account comprehensive bacterial signatures, as proposed in this study, seem therefore reasonable. However, the machine learning was constrained by the small sample size, the lack of an external validation data set, and possible overfitting. The bacterial signature of psychological distress can therefore not claim generalization. It can only help orienting research toward relevant bacteria.

Microbial Diversity and Composition

The bacterial diversity and the number of reads per sample in this study were high in comparison with other studies (62,63). Diversity was however not different between the subgroups, which may be due to the small sample size. Because several animal studies have reported decreased alpha diversity after exposure to stress (15,16,103), further investigations are required to assess the association between psychological variables and alpha diversity in humans. Microbiome composition was associated with psychological distress and depression, whereas other potentially confounding variables showed no association. This indicates systematic shifts in certain taxa in parallel with psychological burden. The cohort was separated in two clusters according to microbial composition. Psychological characteristics were equally distributed among these, with a slightly higher presence of psychological distress in the cluster with lower *Firmicutes* to *Bacteroidetes* ratio. This is similar to the results of Jeffery and colleagues (66), where psychological burden was higher in patients belonging to a cluster with microbiomes resembling those of healthy controls. The authors elegantly interpreted this discovery as a more "centrally triggered" IBS. In contrast to their work, our study lacks a control group for comparison with healthy microbiomes.

Irrespective of microbial analyses, the low correlation between IBS symptom burden and psychological distress was a surprising finding of this study. Previous studies reported mixed magnitudes of this relationship (104–106). In our opinion, the findings reflect that IBS is a heterogeneous disorder (43,45) and that association of psychological and IBS symptoms can occur in any configuration.

Differential Abundance of Specific Taxa

Higher abundances of several bacteria belonging to the two major phyla *Proteobacteria* and *Bacteroidetes* were found in patients with psychological distress. Elevated *Proteobacteria* were previously

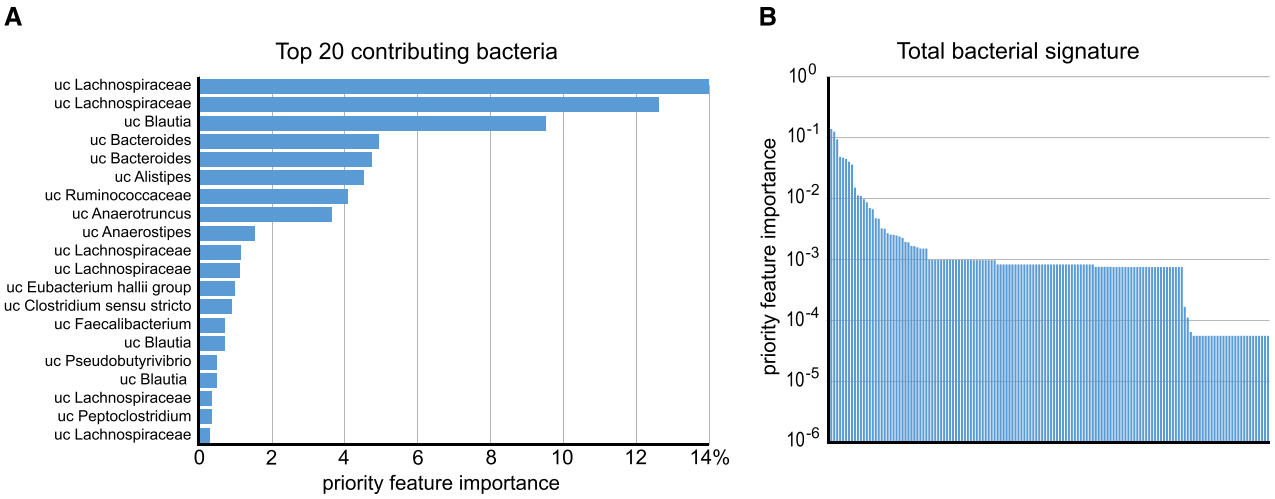

FIGURE 5. Top 20 OTUs with the highest contribution to classifier (A). The lowest known level of taxonomy is given to denote phylogenetic membership, as all top 20 OTUs contributing to the model were unclassified (uc) species. The ranked list of all 148 signature OTUs contributing to classification of psychological distress with logarithmic indication of their contribution to the model (B). Color image is available only in online version (www.psychosomaticmedicine.org).

reported in people with major depressive disorder (40) and as a feature of IBS (58). *Bacteroidetes* members demonstrated a strong presence not only in anxiety (families *Bacteroidaceae* and *Rikenellaceae*) but also in depression (family *Prevotellaceae*). Anxiety correlated positively with the genus *Anaerotruncus*, which was previously found to be increased in animals after prenatal stress (107). A recent study examining IBS and comorbid depression also reported elevated *Bacteroidetes* (70), but studies show mixed results regarding their presence in IBS and in major depression alone (40,62,63,108).

IBS symptom-related variables were also taken into consideration in the microbial subgroup analyses. The severity of IBS was correlated with *Ruminococcus*, which adds to previous findings (58,109). IBS-mix and IBS-C were characterized by elevated *Propionibacterium*. A possible role in slowing down intestinal transit has been previously observed (110) and warrants further investigation. Postinfectious history of IBS onset was associated with a significantly elevated *Erysipelotrichaceae* genus. *Erysipelotrichaceae* were previously found to flourish after treatment with broad-spectrum antibiotics and are rated as highly immunogenic (111). These properties might offer an explanation for subsequent bowel dysfunction after gastrointestinal infections. However, other previously described microbial characteristics of PI-IBS (67,109) were not replicated in our study.

Machine Learning Signature

A comprehensive gut microbial pattern associated with psychological distress was identified with machine learning techniques. Machine learning algorithms are increasingly used to model phenotype-microbe associations (112) or to distinguish between different types of individuals (113,114). Their strength lies in their ability to take into account the complex and interactional nature (115) of the microbiome by simultaneous consideration of several bacterial features and their use in microbiome studies has been encouraged by expert panels (94,116). Machine learning identified associations with psychological distress in a signature of 148 unclassified species, mostly members of the families *Lachnospiraceae* and *Ruminococcaceae*.

CONCLUSIONS

This study assessed relationships between gut microbiota and psychological variables in a sample of patients with IBS. Notably, the study generated further evidence for a relationship between psyche and gut bacteria, underlining the importance of brain-gut alterations and the psychological dimension in IBS. Psychological distress was associated with gut microbiota composition, and a microbial signature corresponding with psychological distress was identified. In-depth characterization of these bacteria might lead to discovery of new biomarkers and therapeutics. The findings further emphasize the relevance of gut bacteria for stress reactivity in humans and for integrated approaches of clinical management of IBS. Future studies will also have to determine, if distress-associated microbial alterations are specific to IBS, a disease picture with both altered microbiome and stress reactivity characteristics, or if similar associations are also present in healthy individuals with varying levels of distress.

The authors thank Christoph Högenauer, Slave Trajanoski, Ingeborg Klymiuk, the Center for Medical Research Graz, Nina Rittershaus, Nicola Stephanou-Rieser, Stella Held, Sarah Russegger, Benjamin Block, and the participants for providing samples and information for the study.

Source of Funding and Conflicts of Interest: The study was supported by Österreichische Nationalbank Jubliäumsfonds (Grant Number 16506), Österreichische Gesellschaft für Gastroenterologie und Hepatologie M.T. has been on the Adisory Boards of Albireo, Gilead, Falk, Novartis, Intercept, MSD, and Phenex. He has received grants to the Medical University of Vienna from Albireo, Falk, Intercept, MSD, and Takeda. He has been on the speakers bureaus for Gilead, Falk, and MSD. He is holding patents for the use of nor-Ursodeoxycholic acid. He has received travel expenses from Gilead and Falk. J.P. has received travel expenses from Yakult. G.M. has been on the Advisory Boards of Allergan and Almirall, she has received grants to the Medical University of Vienna by AbbVie, Vifor, Almirall, Merck, Falk, Yakult, Sanova, Danone, and she has been on the speakers bureaus for Falk, Peri Consulting, Henrich Communication, Milton Erickson Institut Austria, Wirtschaftskammer Austria, and Gebro. She has received payments for development of educational presentations by Ärztekammer Austria and travel expenses by Gebro and Falk.

REFERENCES

1. Mayer EA, Knight R, Mazmanian SK, Cryan JF, Tillisch K. Gut microbes and the brain: paradigm shift in neuroscience. J Neurosci 2014;34:15490–6.
2. Dinan TG, Stilling RM, Stanton C, Cryan JF. Collective unconscious: how gut microbes shape human behavior. J Psychiatr Res 2015;63:1–9.
3. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol 2015;28:203–9.
4. Foster JA, Rinaman L, Cryan JF. Stress & the gut-brain axis: regulation by the microbiome. Neurobiol Stress 2017;124–36.
5. Dinan TG, Cryan JF. Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology 2012; 37:1369–78.
6. Rea K, Dinan TG, Cryan JF. The brain-gut axis contributes to neuroprogression in stress-related disorders. Mod Trends Pharmacopsychiatry 2017;31:152–61.
7. Liu RT. The microbiome as a novel paradigm in studying stress and mental health. Am Psychol 2017;72:655.
8. Wiley N, Dinan T, Ross R, Stanton C, Clarke G, Cryan J. The microbiota-gut-brain axis as a key regulator of neural function and the stress response: implications for human and animal health. J Anim Sci 2017;95:3225–46.
9. Mayer EA, Naliboff BD, Chang L, Coutinho SV. V. Stress and irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2001;280:G519–G24.
10. Mayer EA, Savidge T, Shulman RJ. Brain–gut microbiome interactions and functional bowel disorders. Gastroenterology 2014;146:1500–12.
11. Vanner SJ, Greenwood-Van Meerveld B, Mawe GM, Shea-Donohue T, Verdu EF, Wood J, Grundy D. Fundamentals of neurogastroenterology: basic science. Gastroenterology 2016;150:1280–91.
12. Maier KJ, al'Absi M. Toward a biopsychosocial ecology of the human microbiome, brain-gut axis, and health. Psychosom Med 2017;79:947–57.
13. Tache Y, Larauche M, Yuan P-Q, Million M. Brain and gut CRF signaling: biological actions and role in the gastrointestinal tract. Curr Mol Pharmacol 2018;11:51–71.
14. Bhatia V, Tandon RK. Stress and the gastrointestinal tract. J Gastroenterol Hepatol 2005;20:332–9.
15. Bharwani A, Mian MF, Foster JA, Surette MG, Bienenstock J, Forsythe P. Structural & functional consequences of chronic psychosocial stress on the microbiome & host. Psychoneuroendocrinology 2016;63:217–27.
16. Galley JD, Nelson MC, Yu Z, Dowd SE, Walter J, Kumar PS, Lyte M, Bailey MT. Exposure to a social stressor disrupts the community structure of the colonic mucosa-associated microbiota. BMC Microbiol 2014;14:189.
17. Bangsgaard Bendtsen KM, Krych L, Sørensen DB, Pang W, Nielsen DS, Josefsen K, Hansen LH, Sørensen SJ, Hansen AK. Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. PLoS One 2012;7:e46231.
18. Dunphy-Doherty F, O'Mahony SM, Peterson VL, O'Sullivan O, Crispie F, Cotter PD, Wigmore P, King MV, Cryan JF, Fone KCF. Post-weaning social isolation of rats leads to long-term disruption of the gut microbiota-immune-brain axis. Brain Behav Immun 2018;68:261–73.

19. Jašarević E, Howard CD, Misic AM, Beiting DP, Bale TL. Stress during pregnancy alters temporal and spatial dynamics of the maternal and offspring microbiome in a sex-specific manner. Sci Rep 2017;7:44182.
20. Moloney RD, O'Mahony SM, Dinan TG, Cryan JF. Stress-induced visceral pain: toward animal models of irritable-bowel syndrome and associated comorbidities. Front Psychiatry 2015;68:335–42.
21. Fourie NH, Wang D, Abey SK, Creekmore AL, Hong S, Martin CG, Wiley JW, Henderson WA. Structural and functional alterations in the colonic microbiome of the rat in a model of stress induced irritable bowel syndrome. Gut Microbes 2017;8:33–45.
22. De Palma G, Lynch MD, Lu J, Dang VT, Deng Y, Jury J, Umeh G, Miranda PM, Pigrau Pastor M, Sidani S, Pinto-Sanchez MI, Philip V, McLean PG, Hagelsieb MG, Surette MG, Bergonzelli GE, Verdu EF, Britz-McKibbin P, Neufeld JD, Collins SM, Bercik P. Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice. Sci Transl Med 2017;9:379.
23. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J Physiol 2004;558:263–75.
24. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 2011;23:255–64.
25. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, Dinan TG, Cryan JF. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 2013; 18:666–73.
26. Tsilimigras MC, Gharaibeh RZ, Sioda M, Gray L, Fodor AA, Lyte M. Interactions between stress and sex in microbial responses within the microbiota-gut-brain axis in a mouse model. Psychosom Med 2018;80:361–9.
27. Cowan CSM, Hoban AE, Ventura-Silva AP, Dinan TG, Clarke G, Cryan JF. Gutsy moves: the amygdala as a critical node in microbiota to brain signaling. Bioessays 2018;40.
28. Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA, Fahnestock M, Moine D, Berger B, Huizinga JD, Kunze W, McLean PG, Bergonzelli GE, Collins SM, Verdu EF. The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil 2011;23:1132–9.
29. Schulz A, Vögele C. Interoception and stress. Front Psychol 2015;6:993.
30. Zaneveld JR, McMinds R, Vega Thurber R. Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nat Microbiol 2017;2:17121.
31. Kennedy PJ, Cryan JF, Dinan TG, Clarke G. Kynurenine pathway metabolism and the microbiota-gut-brain axis. Neuropharmacology 2017;112:399–412.
32. Morris G, Berk M, Carvalho A, Caso JR, Sanz Y, Walder K, Maes M. The role of the microbial metabolites including tryptophan catabolites and short chain fatty acids in the pathophysiology of immune-inflammatory and neuroimmune disease. Mol Neurobiol 2017;54:4432–51.
33. Stilling RM, Dinan TG, Cryan JF. Microbial genes, brain & behaviour - epigenetic regulation of the gut-brain axis. Genes Brain Behav 2014;13:69–86.
34. Gacias M, Gaspari S, Santos PM, Tamburini S, Andrade M, Zhang F, Shen N, Tolstikov V, Kiebish MA, Dupree JL, Zachariou V, Clemente JC, Casaccia P. Microbiota-driven transcriptional changes in prefrontal cortex override genetic differences in social behavior. Elife 2016;5:e13442.
35. Kiraly DD, Walker DM, Calipari ES, Labonte B, Issler O, Pena CJ, Ribeiro EA, Russo SJ, Nestler EJ. Alterations of the host microbiome affect behavioral responses to cocaine. Sci Rep 2016;6:35455.
36. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, Guyonnet D, Legrain-Raspaud S, Trotin B, Naliboff B, Mayer EA. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology 2013;144:1394–401.
37. Tillisch K, Mayer EA, Gupta A, Gill Z, Brazeilles R, Le Nevé B, van Hylckama Vlieg JET, Guyonnet D, Derrien M, Labus JS. Brain structure and response to emotional stimuli as related to gut microbial profiles in healthy women. Psychosom Med 2017;79:905–13.
38. Labus J, Oezguen N, Hollister EB, Tillisch K, Savidge T, Versalovic J. Regional brain morphology is associated with gut microbial metabolites in irritable bowel syndrome (IBS). Gastroenterology 2015;148.
39. Kim H, Park Y-J. The association between temperament and microbiota in healthy individuals: a pilot study. Psychosom Med 2017;79:898–904.
40. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, Wang W, Tang W, Tan Z, Shi J, Li L, Ruan B. Altered fecal microbiota composition in patients with major depressive disorder. Brain Behav Immun 2015;48:186–94.
41. Naseribafrouei A, Hestad K, Avershina E, Sekelja M, Linløkken A, Wilson R, Rudi K. Correlation between the human fecal microbiota and depression. Neurogastroenterol Motil 2014;26:1155–62.
42. Vuong HE, Hsiao EY. Emerging roles for the gut microbiome in autism spectrum disorder. Biol Psychiatry 2017;81:411–23.
43. Sundin J, Öhman L, Simren M. Understanding the Gut Microbiota in Inflammatory and Functional Gastrointestinal Diseases. Psychosom Med 2017;79: 857–67.
44. Drossman DA, Hasler WL. Rome IV—functional GI disorders: disorders of gut-brain interaction. Gastroenterology 2016;150:1257–61.
45. Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology 2002;122:1140–56.
46. Fond G, Loundou A, Hamdani N, Boukouaci W, Dargel A, Oliveira J, Roger M, Tamouza R, Leboyer M, Boyer L. Anxiety and depression comorbidities in irritable bowel syndrome (IBS): a systematic review and meta-analysis. Eur Arch Psychiatry Clin Neurosci 2014;264:651–60.
47. Kennedy PJ, Cryan JF, Quigley EM, Dinan TG, Clarke G. A sustained hypothalamic-pituitary-adrenal axis response to acute psychosocial stress in irritable bowel syndrome. Psychol Med 2014;44:3123–34.
48. Park SH, Naliboff BD, Shih W, Presson AP, Videlock EJ, Ju T, Kilpatrick L, Gupta A, Mayer EA, Chang L. Resilience is decreased in irritable bowel syndrome and associated with symptoms and cortisol response. Neurogastroenterol Motil 2018;30.
49. Kano M, Muratsubaki T, Van Oudenhove L, Morishita J, Yoshizawa M, Kohno K, Yagihashi M, Tanaka Y, Mugikura S, Dupont P, Ly HG, Takase K, Kanazawa M, Fukudo S. Altered brain and gut responses to corticotropin-releasing hormone (CRH) in patients with irritable bowel syndrome. Sci Rep 2017;7:12425.
50. Choghakhori R, Abbasnezhad A, Hasanvand A, Amani R. Inflammatory cytokines and oxidative stress biomarkers in irritable bowel syndrome: association with digestive symptoms and quality of life. Cytokine 2017;93:34–43.
51. Bennet SM, Polster A, Törnblom H, Isaksson S, Capronnier S, Tessier A, Le Nevé B, Simrén M, Öhman L. Global cytokine profiles and association with clinical characteristics in patients with irritable bowel syndrome. Am J Gastroenterol 2016;111:1165–76.
52. Yoon H. Mast Cell May Be the Master Key to Solve the Mystery of Pathogenesis of Irritable Bowel Syndrome. Gut Liver 2016;10:325–6.
53. Keszthelyi D, Troost FJ, Jonkers DM, van Eijk HM, Lindsey PJ, Dekker J, Buurman WA, Masclee AA. Serotonergic reinforcement of intestinal barrier function is impaired in irritable bowel syndrome. Aliment Pharmacol Ther 2014;40:392–402.
54. Valentin N, Camilleri M, Altayar O, Vijayvargiya P, Acosta A, Nelson AD, Murad MH. Biomarkers for bile acid diarrhoea in functional bowel disorder with diarrhoea: a systematic review and meta-analysis. Gut 2016;65:1951–9.
55. Claassen J, Labrenz F, Ernst TM, Icenhour A, Langhorst J, Forsting M, Timmann D, Elsenbruch S. Altered cerebellar activity in visceral pain-related fear conditioning in irritable bowel syndrome. Cerebellum 2017;16:508–17.
56. Schmid J, Langhorst J, Gaß F, Theysohn N, Benson S, Engler H, Gizewski ER, Forsting M, Elsenbruch S. Placebo analgesia in patients with functional and organic abdominal pain: a fMRI study in IBS, UC and healthy volunteers. Gut 2015;64:418–27.
57. Simrén M, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG; Rome Foundation Committee. Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut 2013;62: 159–76.
58. Rajilić-Stojanović M, Jonkers DM, Salonen A, Hanevik K, Raes J, Jalanka J, De Vos WM, Manichanh C, Golic N, Enck P, Philippou E, Iraqi FA, Clarke G, Spiller RC, Penders J. Intestinal microbiota and diet in IBS: causes, consequences, or epiphenomena? Am J Gastroenterol 2015;110:278–87.
59. Ringel Y, Ringel-Kulka T. The intestinal microbiota and irritable bowel syndrome. J Clin Gastroenterol 2015;49:S56–S9.
60. Moser G, Fournier C, Peter J. Intestinal microbiome-gut-brain axis and irritable bowel syndrome. Wien Med Wochenschr 2018;168:62–6.
61. Rajilić-Stojanović M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 2011;141:1792–801.
62. Pozuelo M, Panda S, Santiago A, Mendez S, Accarino A, Santos J, Guarner F, Azpiroz F, Manichanh C. Reduction of butyrate-and methane-producing microorganisms in patients with irritable bowel syndrome. Sci Rep 2015;5:12693.
63. Tap J, Derrien M, Törnblom H, Brazeilles R, Cools-Portier S, Doré J, Störsrud S, Le Nevé B, Öhman L, Simrén M. Identification of an intestinal microbiota signature associated with severity of irritable bowel syndrome. Gastroenterology 2017;152:111–23.
64. Gargari G, Taverniti V, Gardana C, Cremon C, Canducci F, Pagano I, Barbaro MR, Bellacosa L, Castellazzi AM, Valsecchi C, Tagliacarne SC, Bellini M, Bertani L, Gambaccini D, Marchi S, Cicala M, Germanà B, Dal Pont E, Vecchi M, Ogliari C, Fiore W, Stanghellini V, Barbara G, Guglielmetti S. Fecal Clostridiales distribution and short-chain fatty acids reflect bowel habits in irritable bowel syndrome. Environ Microbiol 2018.
65. Ringel-Kulka T, Choi CH, Temas D, Kim A, Maier DM, Scott K, Galanko JA, Ringel Y. Altered colonic bacterial fermentation as a potential pathophysiological factor in irritable bowel syndrome. Am J Gastroenterol 2015;110:1339.
66. Jeffery IB, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 2012;61:997–1006.
67. Sundin J, Rangel I, Fuentes S, Heikamp-de Jong I, Hultgren-Hörnquist E, de Vos WM, Brummer RJ. Altered faecal and mucosal microbial composition in post-infectious irritable bowel syndrome patients correlates with mucosal lymphocyte phenotypes and psychological distress. Aliment Pharmacol Ther 2015;41:342–51.

68. Labus JS, Hollister EB, Jacobs J, Kirbach K, Oezguen N, Gupta A, Acosta J, Luna RA, Aagaard K, Versalovic J, Savidge T, Hsiao E, Tillisch K, Mayer EA. Differences in gut microbial composition correlate with regional brain volumes in irritable bowel syndrome. Microbiome 2017;5:49.
69. Aizawa E, Tsuji H, Asahara T, Takahashi T, Teraishi T, Yoshida S, Ota M, Koga N, Hattori K, Kunugi H. Possible association of Bifidobacterium and Lactobacillus in the gut microbiota of patients with major depressive disorder. J Affect Disord 2016;202:254–7.
70. Liu Y, Zhang L, Wang X, Wang Z, Zhang J, Jiang R, Wang X, Wang K, Liu Z, Xia Z. Similar fecal microbiota signatures in patients with diarrhea-predominant irritable bowel syndrome and patients with depression. Clin Gastroenterol Hepatol 2016;14:1602–11. e5.
71. Petermann F. Hospital Anxiety and Depression Scale, Deutsche Version (HADS-D). Z Klin Psychol Psychiatr Psychother 2015;59:251–3.
72. Herrmann-Lingen C, Buss U, Snaith P. Hospital Anxiety and Depression Scale-Deutsche Version (HADS-D). Bern: Huber; 2011.
73. Sellick SM, Edwardson AD. Screening new cancer patients for psychological distress using the hospital anxiety and depression scale. Psychooncology 2007;16:534–42.
74. Fliege H, Rose M, Arck P, Walter OB, Kocalevent R-D, Weber C, Klapp BF. The Perceived Stress Questionnaire (PSQ) reconsidered: validation and reference values from different clinical and healthy adult samples. Psychosom Med 2005;67:78–88.
75. Fliege H, Rose M, Arck P, Levenstein S, Klapp BF. Validierung des "perceived stress questionnaire"(PSQ) an einer deutschen Stichprobe. [Validation of the "Perceived Stress Questionnaire"(PSQ) in a German sample.]. Diagnostica 2001;47:142–52.
76. Kocalevent R-D, Levenstein S, Fliege H, Schmid G, Hinz A, Brähler E, Klapp BF. Contribution to the construct validity of the Perceived Stress Questionnaire from a population-based survey. J Psychosom Res 2007;63:71–81.
77. Moser G, Trägner S, Gajowniczek EE, Mikulits A, Michalski M, Kazemi-Shirazi L, Kulnigg-Dabsch S, Führer M, Ponocny-Seliger E, Dejaco C, Miehsler W. Long-term success of GUT-directed group hypnosis for patients with refractory irritable bowel syndrome: a randomized controlled trial. Am J Gastroenterol 2013;108:602–9.
78. Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. Aliment Pharmacol Ther 1997;11:395–402.
79. Betz C, Mannsdörfer K, Bischoff S. Validation of the IBS-SSS. Z Gastroenterol 2013;51:1171–6.
80. Spiegel B, Camilleri M, Bolus R, Andresen V, Chey WD, Fehnel S, Mangel A, Talley NJ, Whitehead WE. Psychometric evaluation of patient-reported outcomes in irritable bowel syndrome randomized controlled trials: a Rome Foundation report. Gastroenterology 2009;137:1944–53. e3.
81. Mujagic Z, Keszthelyi D, Aziz Q, Reinisch W, Quetglas EG, De Leonardis F, Segerdahl M, Masclee AA. Systematic review: instruments to assess abdominal pain in irritable bowel syndrome. Aliment Pharmacol Ther 2015;42:1064–81.
82. Klymiuk I, Bambach I, Patra V, Trajanoski S, Wolf P. 16S based microbiome analysis from healthy subjects' skin swabs stored for different storage periods reveal phylum to genus level changes. Front Microbiol 2016;7.
83. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590–D6.
84. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.
85. Fisher RA. The use of multiple measurements in taxonomic problems. Ann Eugen 1936;7:179–88.
86. Knights D, Costello EK, Knight R. Supervised classification of human microbiota. FEMS Microbiol Rev 2011;35:343–59.
87. Breiman L. Random forests. Machine Learning 2001;45:5–32.
88. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6.
89. Goecks J, Nekrutenko A, Taylor J; Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome Biol 2010;11:R86.
90. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2017.
91. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI. Human gut microbiome viewed across age and geography. Nature 2012;486:222–7.
92. Jašarević E, Morrison KE, Bale TL. Sex differences in the gut microbiome–brain axis across the lifespan. Phil Trans R Soc B 2016;371:20150122.
93. Le Bastard Q, Al-Ghalith GA, Grégoire M, Chapelet G, Javaudin F, Dailly E, Batard E, Knights D, Montassier E. Systematic review: human gut dysbiosis induced by non-antibiotic prescription medications. Aliment Pharmacol Ther 2017;47:332–45.
94. Camacho DM, Collins KM, Powers RK, Costello JC, Collins JJ. Next-generation machine learning for biological networks. Cell 2018;173:1581–92.
95. Coyne JC, van Sonderen E. No further research needed: abandoning the Hospital and Anxiety Depression Scale (HADS). J Psychosom Res 2012;72:173–4.
96. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26:2460–1.
97. Koskinen K, Auvinen P, Björkroth KJ, Hultman J. Inconsistent denoising and clustering algorithms for amplicon sequence data. J Comput Biol 2015;22:743–51.
98. Westcott SL, Schloss PD. OptiClust, an improved method for assigning amplicon-based sequence data to operational taxonomic units. mSphere 2017;2:e00073–17.
99. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Caporaso JG. Optimizing taxonomic classification of marker gene amplicon sequences. PeerJ Preprints 2018;2167–9843.
100. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J 2017;11:2639–43.
101. Wiest R, Albillos A, Trauner M, Bajaj J, Jalan R. Targeting the gut-liver axis in liver disease. J Hepatol 2017;68:1084–103.
102. Eren AM, Maignien L, Sul WJ, Murphy LG, Grim SL, Morrison HG, Sogin ML. Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data. Methods Ecol Evol 2013;4:1111–9.
103. Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 2011;25:397–407.
104. Pinto-Sanchez MI, Ford AC, Avila CA, Verdu EF, Collins SM, Morgan D, Moayyedi P, Bercik P. Anxiety and depression increase in a stepwise manner in parallel with multiple FGIDs and symptom severity and frequency. Am J Gastroenterol 2015;110:1038.
105. Knowles SR, Austin DW, Sivanesan S, Tye-Din J, Leung C, Wilson J, Castle D, Kamm MA, Macrae F, Hebbard G. Relations between symptom severity, illness perceptions, visceral sensitivity, coping strategies and well-being in irritable bowel syndrome guided by the common sense model of illness. Psychol Health Med 2017;22:524–34.
106. Lackner JM, Gudleski GD, Thakur ER, Stewart TJ, Iacobucci GJ, Spiegel BM. The impact of physical complaints, social environment, and psychological functioning on IBS patients' health perceptions: looking beyond GI symptom severity. Am J Gastroenterol 2014;109:224–33.
107. Golubeva AV, Crampton S, Desbonnet L, Edge D, O'Sullivan O, Lomasney KW, Zhdanov AV, Crispie F, Moloney RD, Borre YE, Cotter PD, Hyland NP, O'Halloran KD, Dinan TG, O'Keeffe GW, Cryan JF. Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. Psychoneuroendocrinology 2015;60:58–74.
108. Zheng P, Zeng B, Zhou C, Liu M, Fang Z, Xu X, Zeng L, Chen J, Fan S, Du X, Zhang X, Yang D, Yang Y, Meng H, Li W, Melgiri ND, Licinio J, Wei H, Xie P. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. Mol Psychiatry 2016;21:786–96.
109. Jalanka-Tuovinen J, Salojarvi J, Salonen A, Immonen O, Garsed K, Kelly FM. Faecal microbiota composition and host-microbe cross-talk following gastroenteritis and in postinfectious irritable bowel syndrome. Gut 2014;63.
110. Bougle D, Roland N, Lebeurrier F, Arhan P. Effect of propionibacteria supplementation on fecal bifidobacteria and segmental colonic transit time in healthy human subjects. Scand J Gastroenterol 1999;34:144–8.
111. Kaakoush NO. Insights into the role of erysipelotrichaceae in the human host. Front Cell Infect Microbiol 2015;5:84.
112. Thaiss CA, Itav S, Rothschild D, Meijer M, Levy M, Moresi C, Dohnalová L, Braverman S, Rozin S, Malitsky S, Dori-Bachash M, Kuperman Y, Biton I, Gertler A, Harmelin A, Shapiro H, Halpern Z, Aharoni A, Segal E, Elinav E. Persistent microbiome alterations modulate the rate of post-dieting weight regain. Nature 2016;540:544–51.
113. Giloteaux L, Goodrich JK, Walters WA, Levine SM, Ley RE, Hanson MR. Reduced diversity and altered composition of the gut microbiome in individuals with myalgic encephalomyelitis/chronic fatigue syndrome. Microbiome 2016;4:30.
114. Papa E, Docktor M, Smillie C, Weber S, Preheim SP, Gevers D, Giannoukos G, Ciulla D, Tabbaa D, Ingram J, Schauer DB, Ward DV, Korzenik JR, Xavier RJ, Bousvaros A, Alm EJ. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. PLoS One 2012;7:e39242.
115. Falony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, Kurilshikov A, Bonder MJ, Valles-Colomer M, Vandeputte D, Tito RY, Chaffron S, Rymenans L, Verspecht C, De Sutter L, Lima-Mendez G, D'hoe K, Jonckheere K, Homola D, Garcia R, Tigchelaar EF, Eeckhaudt L, Fu J, Henckaerts L, Zhernakova A, Wijmenga C, Raes J. Population-level analysis of gut microbiome variation. Science 2016;352:560–4.
116. Aroniadis OC, Drossman DA, Simrén M. A Perspective on Brain–Gut Communication: The American Gastroenterology Association and American Psychosomatic Society Joint Symposium on Brain–Gut Interactions and the Intestinal Microenvironment. Psychosom Med 2017;79:847–56.