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Correlation between the human fecal microbiota and depression

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Key Messages

- We found a significant correlation between the gut microbiota and depression in humans. The correlations, however, were in opposite directions for closely related Operational Taxonomic Units (OTU's).
- The aim of our work was to investigate the correlation between the human fecal microbiota and depression.
- 16S rRNA gene Illumina deep sequencing from 37 depressed and 18 non-depressed individuals was conducted. The deep sequencing data were analyzed using an in-house generated computer program, enabling the resolution of closely related OTU's.
- The order *Bacteroidales* was overrepresented ($p = 0.05$), while the family *Lachnospiraceae* was underrepresented ($p = 0.02$) with respect to OTU's associated with depression.

Abstract

Background *Depression is a chronic syndrome with a pathogenesis linked to various genetic, biological, and environmental factors. Several links between gut microbiota and depression have been established in animal models. In humans, however, few correlations have yet been demonstrated. The aim of our work was therefore to identify potential correlations between human fecal microbiota (as a proxy for gut microbiota) and depression.* ***Methods*** *We analyzed fecal samples from 55 people, 37 patients, and 18 non-depressed controls. Our analyses were based on data generated by Illumina deep sequencing of 16S rRNA gene amplicons.* ***Key Results*** *We found several correlations between depression and fecal microbiota. The correlations, however, showed opposite directions even for closely related Operational Taxonomic Units (OTU's), but were still associated with certain higher order phylogroups. The order Bacteroidales showed an overrepresentation ($p = 0.05$), while the family Lachnospiraceae showed an underrepresentation ($p = 0.02$) of OTU's associated with depression. At low taxonomic levels, there was one clade consisting of five OTU's within the genus Oscillibacter, and one clade within Alistipes (consisting of four OTU's) that showed a significant association with depression ($p = 0.03$ and 0.01, respectively).* ***Conclusions & Inferences*** *The Oscillibacter type strain has valeric acid as its main metabolic end product, a homolog of neurotransmitter GABA, while Alistipes has previously been shown to be associated with induced stress in mice. In conclusion, the taxonomic correlations detected here may therefore correspond to mechanistic models.*

Keywords *16SrRNA gene, depression, gut microbiota, Illumina deep sequencing.*

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INTRODUCTION

Major depressive disorder is a mental disorder which presents itself with low mood, low self-esteem, and loss of interest in normally enjoyable activities.[1]

Depression is a multi-factorial disease being caused by biological, psychological, and social factors. The diathesis–stress model proposes that depression is caused when stressful life events superimpose on a pre-existing vulnerable condition.[2]

There has been recent major interest in the gut–brain axis in relation to depression. Three general mechanisms have been suggested to describe how the gut microbiota influences depression, namely through inflammation, the Hypothalamic–Pituitary–Adrenal axis (HPA), or interference with neurotransmitter signaling.[3]

IgA- and IgM-mediated inflammatory responses to lipopolysaccharide have been shown to be elevated in depressed patients.[4] It has also been suggested that depression can result from maladaptation due to abnormalities in circulating cytokines.[5,6] A meta-analysis of the clinical literature implicates higher inflammatory IL-6 and TNF-α in depressed patients compared to controls.[7] Furthermore, in mice it has been shown that gastrointestinal inflammation both induces anxiety behavior and alters central nervous system biochemistry.[8]

HPA is a neuro-endocrine stress response system, being important in both mood disorders and functional diseases. Alterations of the HPA system have been diagnosed in patients harboring different mental states including posttraumatic stress disorder,[9] schizophrenia,[10] social anxiety[11], and depression.[12] In a rat model, it has recently been shown that treatment with probiotic bacteria can interfere with the HPA response to acute physiological stress,[13] suggesting a mechanistic connection between the gut microbiota, HPA, and stress.

Direct interference with neurotransmitter signaling may also be involved in depressive disorders. It has been shown that the neurotransmitter GABA can be produced by intestinal bacteria.[14] Furthermore, probiotic bacteria can modulate depressive behavior through GABA signaling in a mouse model.[15] The other signaling pathway that has been linked to depression is serotonergic signaling, where it has been shown that the serotonergic turnover is higher in the striatum in germ-free mice compared to conventional animals.[16]

Despite several indirect lines of evidence correlating gut microbiota to depression through inflammatory, stress, or signaling pathways, we still lack knowledge about the direct correlation patterns between gut microbiota and depression in humans.[3,17] Therefore, our study aims at identifying direct correlations between human fecal microbiota (as a proxy for gut microbiota) and depressive disorder.

To elucidate fecal microbiota/depression correlations, we used an explorative approach involving deep Illumina sequencing of 16S rRNA gene amplicons. We analyzed a relatively large cohort of clinically depressed patients (n = 37) and matched non-depressed controls (n = 18). Due to the complexity of the data, we chose a combination of multivariate statistical methods in determining correlations between the fecal microbiota and depression.

MATERIALS AND METHODS

Patients and design

Thirty-seven depressed patients were recruited from an inward and outpatient mental health clinic of the Innlandet Hospital in Norway. They represented a group of patients with mild to severe depression. All participants had a diagnosis of depression according to the research criteria of ICD-10, and using the Montgomery-Åsberg Depression Rating Scale (MADRS), which is a diagnostic questionnaire with 10 items to evaluate the severity of the disorder.[18] On the MADRS, a score from 0 to 7 is normal with no indication of depression. Scores of 7–19 indicate depressive symptoms of milder forms, 20–34 moderate depression, and scores above 34 indicate severe depression. A control group of 18 patients with the same age and gender distribution were recruited from an outpatient neurological unit at the same hospital. These patients had diffuse symptoms, which possibly could be related to cerebral disorders, but no disorders could be found. They all had a careful neurological examination and CT/MRI scans. Descriptive statistics for the cohort are shown in Table 1.

The study was designed as a partially blinded observational study in which clinical information including diagnostic status remained unknown until after fecal microbial analyses were finalized.

Stool samples and DNA extraction

Stool samples were immediately frozen at −20 °C in the patient's home freezer after defecation. The samples were then transported at below zero to centralized −70 °C storage. For the patients who were hospitalized, the samples were taken directly to the centralized freezer. Within 1 month, the stool specimens were weighed, and S.T.A.R. (Stool Transport and Recovery; Roche, Basel, Switzerland) buffer solution was added to each sample at a ratio of ~1 (stool) to 3 (S.T.A.R. buffer). Samples were vortexed to achieve homogenous suspension and then stored at −80 °C before DNA extraction.

The DNA extraction protocol used has previously been extensively validated with respect to CE marking of an irritable bowel syndrome (IBS) dysbiosis test.[19] For DNA extraction, frozen stool samples were first allowed to thaw on ice. Microcentrifuge tubes (2 mL) containing 250-mg glass beads (<106 μm) were filled with a suspension volume of 0.5 mL of the stool sample. To achieve bacterial cell lysis, homogenization was performed using a MagNaLyser (Roche) twice at 2000 rpm for 40 s, with 40 s cooling between runs. The samples were kept cold during the rest phase to avoid DNA degradation due to overheating. This step was followed by centrifugation at 12 300 ***g*** for 5 min. The supernatant lysate solution was then transferred to a new microcentrifuge tube in two replicates (designated parallel A and B) for each of the samples. Fifty microlitres supernatant from the tubes were transferred to a KingFisher 96-well plate and DNA was

Table 1 Clinical characteristics

	N	Mean	SD
Age			
Control	18	46.1	13.9
Depressed	37	49.2	13.9
Blood pressure medication			
Control	18	0.22	0.43
Depressed	37	0.24	0.43
Gender (female, *vs* male)			
Control	18	11	7
Depressed	37	20	17
Systolic blood pressure			
Control	18	131.2	14.0
Depressed	37	133.6	23.8
Diastolic blood pressure			
Control	18	80.4	12.4
Depressed	37	82.1	15.0
Education (year)			
Control	18	13.5	2.4
Depressed	37	12.7	2.8
Mini Mental state			
Control	18	29.1	1.0
Depressed	37	28.4	1.6
MADRS depression score			
Control	18	7.2	4.8
Depressed	37	26.3	7.6
Depression medication			
Control	18	0.06	0.24
Depressed	37	0.73	0.45
Body mass index (BMI)			
Control	18	24.7	3.3
Depressed	36	25.9	4.2

extracted using the Mag™ mini kit (LGC, Middlesex, UK), following the manufacturer's recommendations.

Illumina sequencing

For Illumina sequencing, we used different combinations of forward and reverse primers for each sample to generate 116 libraries. These combinations are presented in the Supplementary text. The PCR was conducted in a 25 μL reaction volume with the following composition: 1.25 U HOT FIREPol® DNA polymerase, 1× HOT FIREPol® buffer B2, 2.5 mM Magnesium dichloride, 0.2 mM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer, and 1 μL template. We used 30 cycles of denaturation (95 °C for 30 s), annealing (50 °C for 1 min), and elongation (72 °C for 45 s).

Because of primer-dimer formation, the PCR products were semiquantified by agarose gel electrophoresis, and all the amplicons were mixed in equimolar concentrations. Finally, the pooled products were purified using E.Z.N.A PCR product purification kit (Omega Bio-Tech, Norcross, GA, USA), and submitted for paired-end 250 bp sequencing on the MiSeq Illumina platform at the Norwegian High Throughput Sequencing Center (UiO, Oslo, Norway).

Data analysis

Due to computational speed and resolution, we used a previously developed word-based approach in OTU identification.[20,21] For these analyses, we only included sequences with an average Phred score >31 (per base error rate <0.001) to avoid the influence of sequence errors. For each sample, 4000 sequences were selected (2000 from each of the two parallels). Parallels with <2000 sequences satisfying the filtering criteria were discharged from the analyses. This number was chosen as a trade-off between amount of information and the number of samples lost. Our alignment-independent, multimer-based approach was used to determine the relatedness between the sequences through principal component analysis. OTU binning was based on a 0.5 × 0.5 interval from the score for the two first principal components. The low interval size was chosen to obtain a high resolution for closely related OTU's. The 100 most dominant OTU's were selected for phylogenetic reconstruction (CLC Genomics Workbench) and taxonomic assignment (RDP database with default settings).

We used false discovery rate corrected permutation testing in determining the significance for the univariate differences detected. Briefly, this involves determining whether the differences detected are larger than those expected by chance. For each OTU, we tested if the difference between the average levels (in percentage) in depressed *vs* non-depressed individuals significantly deviated from 0.

To reveal potential complex multivariate interactions between bacteria for the microbiota correlation with depression, we used partial least square discriminant analysis (PLS-DA). This is a multivariate statistical method for relating a binary response variable (in our case depression) to a large number of explanatory variables (in our case OTU's). The models were cross-validated using a Venetian blind approach splitting the dataset into two, using one half to build the model, and the other half for validation.

For verification, we used the Quantitative Insights Into Microbial Ecology (QIIME) pipeline following the analytical recommendations given in the user homepage (http://qiime.org/1.6.0/tutorials/illumina_overview_tutorial.html) with OTU binning at the 1% level and closed reference OTU searches with modified uclust parameters (https://gist.github.com/gregcaporaso/5952785). The QIIME analyses were run on Amazon Cloud Service using StarCluster to load eight m2.4× large instances and start 80 parallel jobs. For each sample, 6000 sequences were picked (3000 from each parallel). Parallels with <3000 sequence reads were discharged from the analyses.

RESULTS

Library size and characteristics

We obtained a total of 4 910 922 reads that could be assigned to the 116 libraries generated. The average number of reads per library was 42 336. The number of reads per sample is summarized in Fig. S1. The technical quality was evaluated by regression analyses from parallel analyses (replicates A and B) with respect to OTU distribution. These analyses showed very good correspondence between the technical replicates, with an average R^2 value of 0.96 ± 0.04. Comparing samples from different individuals, on the other hand, generally gave low R^2 values (<0.3).

Microbiota composition

We identified a total of 1593 OTU's with a pairwise sequence distance between the 100 most dominant

OTU's (representing 61.6% of the sequence reads) in the range 0.5–40% (Fig. S2). The OTU's were classified using the RDP classifier (Table S1), and related using phylogenetic reconstruction (Fig. 1). The most dominant OTU (OTU1) in our dataset was classified as *B. ovatus*. This OTU represented 4% of all the sequence reads (Fig. 1B), and was identified in 54 of 55 individuals. In accordance with a range of other human fecal microbiota studies, we also found a clear dominance of the phyla *Bacteroidetes* and *Firmicutes* in our cohort (Table S1).

Correlation between microbiota and depression

There were no significant differences between depressed and non-depressed patients with respect to species richness, although there was a slight tendency ($p = 0.09$, *t*-test) of a higher number of OTU's among the depressed (374 ± 56) compared to the non-depressed (351 ± 42) individuals. Neither did we identify any significant differences in α-diversity (Simpson's D index of 39.5 ± 15.9 for depressed *vs* 34.4 ± 19.6 for non-depressed patients).

At a high taxonomic level (Domain), there was an underrepresentation of *Bacteroidales* associated with depression ($p = 0.05$, *t*-test for average difference in OTU ratios in Table S1). At a low taxonomic level (OTU), however, we could not identify any single OTU showing significant correlation with depression after false discovery correction.

As no single OTU showed significant correlation with depression, we used multivariate PLS-DA analyses to investigate whether we could detect correlation patterns for the complete microbiota (Fig. 2A). The multivariate model showed good sensitivity and specificity, correctly predicting 100% of the classified depressed patients and 97% of the classified non-depressed patients (misclassifying only one depressed patient as non-depressed). Furthermore, the confounding factor of depression medication did not seem to influence the model, as all 10 depressed patients that did not receive medication, in addition to the control that received medication, were all correctly classified. The redundancy of data was tested using cross-validation. The cross-validated model showed a sensitivity of 0.86 and a specificity of 0.47 (Fig. 2B). To evaluate the robustness of the model, we also performed separate analyses for the A and B parallels, with subsequent correlations of the loadings. These analyses showed good correspondence (Fig. 2C).

The OTU's determined to be correlating with depression according to the regression model (empirically defined as loadings >0.05 or <−0.05) were not evenly distributed among the different taxa (Fig. 2A). There was a higher number of correlating OTU's than expected by chance in the *Bacteroidetes* phylum ($p = 0.05$, binominal test), while there was underrepresentation in the *Lachnospiraceae* family ($p = 0.003$, binominal test). At lower taxonomic levels, there were clades within the genus *Alistipes* ($p = 0.007$, binominal test), and *Oscillibacter* ($p = 0.03$ binominal test) that showed overrepresentation of correlating OTU's. However, no clades showed uniform positive or negative correlations (Fig. 2A).

In support of the OTU-level correlation, binning the data at the genus level (using QIIME) gave poor PLS-DA classification, with misclassification of 22 of the 55 individuals. Furthermore, the cross-validation revealed no redundancy in the data for this model. Due to the low correlation at the genus level, we also tested the direct correlation between the identified OTU's at the 99% identity level. These analyses showed a cross-validated sensitivity of 0.83 and a specificity of 0.37, with none of the individuals being misclassified.

DISCUSSION

The most pronounced high-level correlation detected in our dataset was a general underrepresentation of *Bacteroidetes* related with depression. In both human and animal studies, low *Bacteroidetes* levels have previously been shown to be associated with obesity.[22] It has been previously suggested that there is a link between obesity and depression through low grade inflammation,[23] while we have recently established a correlation between bacteria and low grade inflammation.[24] In our cohort, however, the BMI was only slightly higher for the depressed patients compared to the controls, so it is unlikely that obesity is a confounding factor in our study.

For the low level taxonomic associations detected, a recent study in which mice were subjected to stress over an extended time period, the genus *Alistipes* was one of the bacterial groups that showed the highest increase in the stressed group.[25] Furthermore, *Alistipes* has been found to be elevated in chronic fatigue syndrome[26] and IBS.[27] It has been suggested that *Alistipes* is associated with inflammation,[26] and therefore potentially linked to depression through inflammatory pathways.[3] For *Oscillibacter*, the type strain of this genus has valeric acid as its main metabolic end product.[28] Valeric acid structurally resembles GABA, and has been shown to bind the GABAa receptor. Therefore, it is possible that bacteria involved in valeric acid production and/or metabolism could also be associated with depression.[29] Knowledge about the

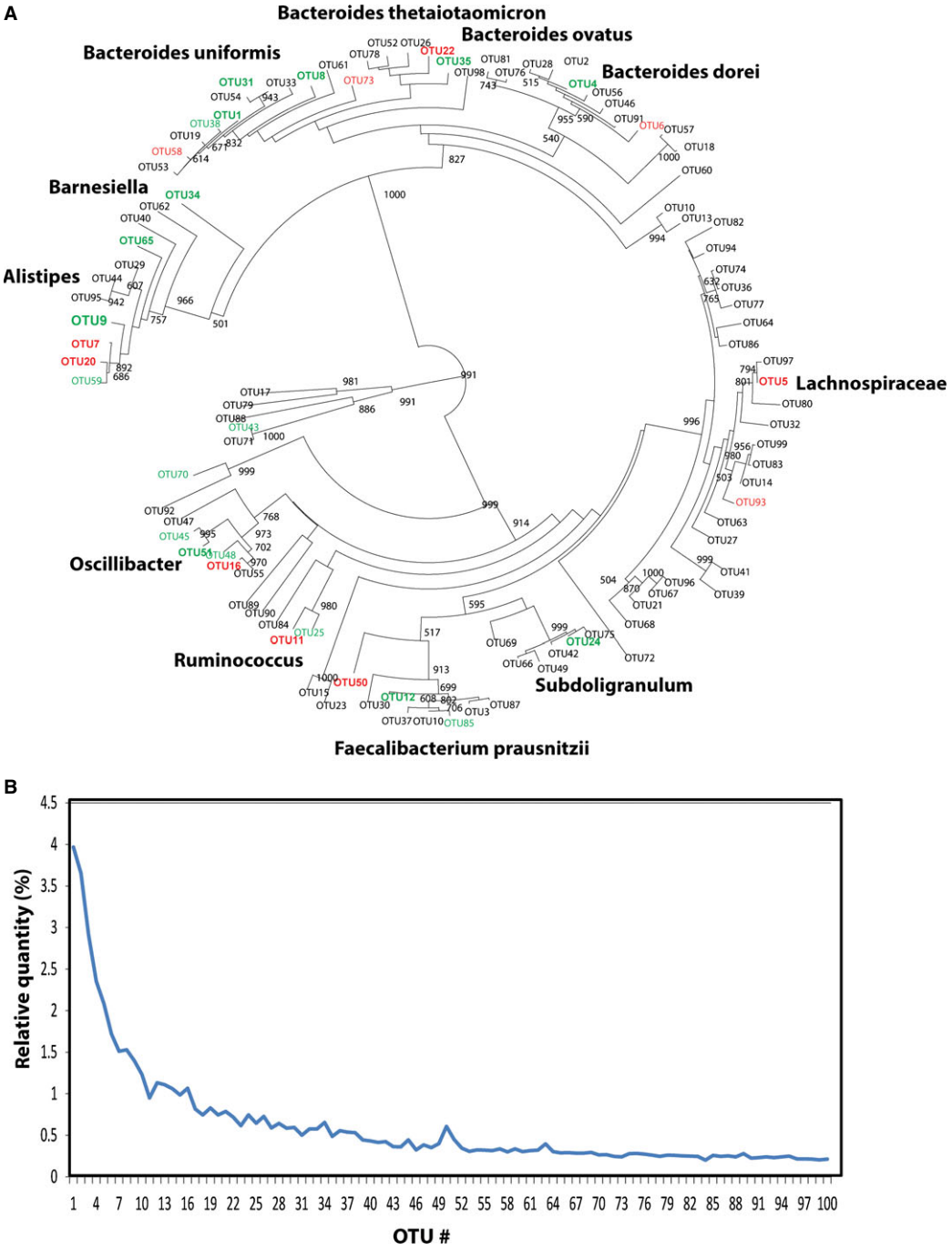

Figure 1 Phylogenetic tree of the 100 most dominant OTU's. (A) The tree was constructed by the neighbor-joining algorithm using bootstrap for testing the support of the branches. The numbers at the nodes show how many out of 1000 bootstrap trees that supported the particular branch (only values >500 are shown). The coloring indicates if the OTU is positively (red) or negatively (green) associated with depression. (B) The ranking of the OTU's by the percentage of the complete dataset is shown.

role of valeric acid in the gut, however, is very sparse, despite constituting one of the main short-chain fatty acids.

The opposite correlations for closely related OTU's in our dataset were both striking and surprising. This may indicate that certain phylogroups interfere with particular mode-modulating pathways, but that the effect of the interference represents a fine tuned balance.[3] Furthermore, the lack of statistically significant univariate correlations may also indicate that

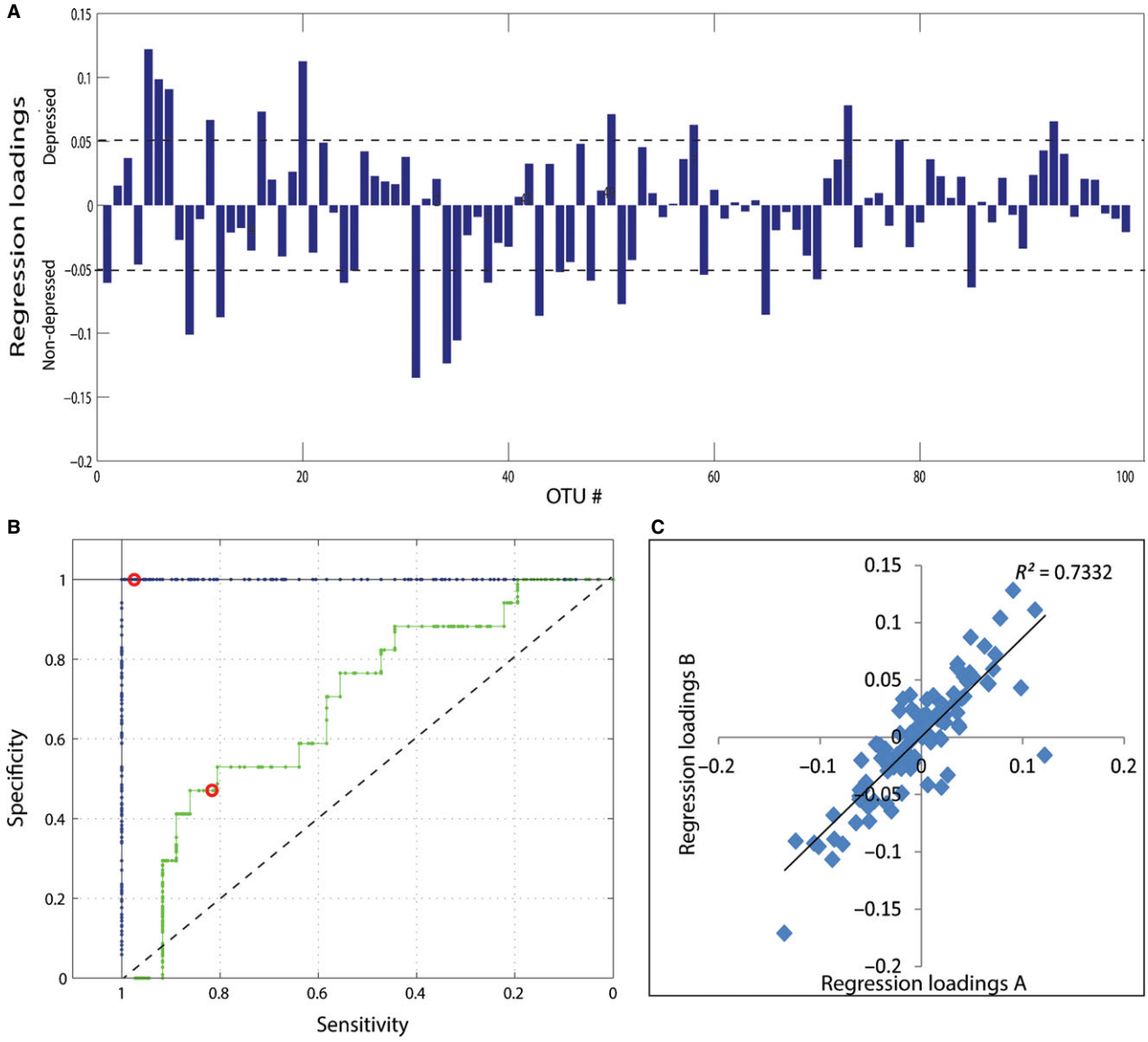

Figure 2 Partial least square discriminant analysis (PLS-DA) classification of depression based on the 100 most dominant OTU's. (A) Loading plot for the correlation of OTU's on depression. The absolute value for correlations >0.5 are marked with a stippled line. (B) ROC plot for the estimated (black) and the cross-validated classification (green). Red circles represent the optimal threshold between specificity and sensitivity. (C) Correlation for the loadings derived from a model built separately from the A and B parallels.

interaction networks are important. These findings, however, must be verified in larger cohorts due to the risk of overseeing effects in small cohorts.

It has previously been shown that both *Alistipes* and *Oscillibacter* levels can be modified through dietary intervention. A diet high in easily fermentable oligo- or mono-saccharides with a low healthy food diversity index promoted the level of *Alistipes*.[30] In a mouse feeding trial, the development of insulin resistance was correlated with reduced levels of *Oscillibacter* following a diet high in fat.[31] Assuming that the correlations detected here contribute to depression, then the potential of modulating depressive disorders through dietary intervention may exist. However, given that bacterial interaction networks are important in shaping the gut microbiota, then the response to interventions may be highly individual, and difficult to predict.

For the factors recorded, medication is confounding with depression. However, as all 10 non-medicated patients, in addition to the medicated control, were correctly classified in both the in-house and QIIME

99% level OTU multivariate models, we believe that it is unlikely that medication in itself can explain the correlation patterns detected. Diet was not logged in this study, but all the patients and controls were ethnic Norwegians most likely consuming a traditional Norwegian diet. The depressed patients were also tended to by medical personnel to ensure proper diet.

Recent evidence has revealed the importance of OTU binning and definitions in discovering biological correlation patterns.[32,33] The results presented here also support the importance of OTU binning at the right taxonomic level, as binning at the genus level did not reveal any correlations, while binning at the 99–99.5% identity level revealed relatively strong correlations between gut microbiota and depression.

In conclusion, we found significant correlations between gut microbiota and depression. The correlations, however, were complex with opposite directions for closely related OTU's.

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CONFLICTS OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTION

AN performed the research; KH designed the study; EA, MS, AL, and KR analyzed the data; KR and RW wrote the article with contribution from all the authors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of number of reads per sample.

Figure S2. Heat map of the pairwise distances for the 100 most dominant OTU's.

Table S1. RDP classification and distribution of the 100 most dominant OTU's.