Fecal microbiota and metabolites are distinct in a pilot study of pediatric Crohn's disease patients with higher levels of perceived stress

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Abstract

Stress is associated with increased Crohn's Disease (CD) activity. This pilot study tested whether pediatric patients with CD reporting higher levels of perceived stress exhibited differences in the fecal microbiome and metabolome. The perceived stress scale (PSS) questionnaire was administered within 2 days of collecting a stool sample for microbiome (using 16S rRNA gene sequencing) and metabolome (using NMR metabolomics) analyses. Higher levels of perceived stress were correlated with increased disease activity on the short Pediatric Crohn's Disease Activity Index (sPCDAI). Patients with High PSS scores vs. Low PSS scores based on a median split had significantly lower relative abundances of Firmicutes and *Anaerostipes*, as well as higher relative abundances of *Parabacteroides*. Fecal alanine and nicotinate were also significantly

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Conflict of Interests

The authors do not have any conflicts of interests that affect the conduct or interpretation of this study.

different in patients with High vs. Low PSS Scores. This pilot study suggests that the fecal microbiome and metabolome differs in pediatric patients with CD and high perceived stress.

1. Introduction

The inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are thought to be due to disrupted homeostasis between the intestinal immune system and the gut microbiota in genetically susceptible individuals (Kostic, Xavier, & Gevers, 2014). Alterations in the microbiota can be both a cause and a consequence of mucosal inflammation, illustrating a complex cycle of host-microbe interactions during IBD, and multiple studies demonstrate abnormal microbial communities in IBD patients (Gevers et al., 2014). The onset and severity of the disease can be heavily influenced by environmental factors, with one environmental factor being exposure to stress (Bernstein et al., 2010; Camara et al., 2009; Traue & Kosarz, 1999). Multiple studies in laboratory animals demonstrate that stressor exposure can change the composition of the gut microbiota (Mackos, Maltz, & Bailey, 2016). However, it is not yet known whether stress is associated with altered gut microbiota composition, and/or function, in pediatric IBD patients.

In adults with IBD, levels of stress, depression, or anxiety are predictors of future disease activity (Bernstein et al., 2010; Bitton et al., 2008; Langhorst, Hofstetter, Wolfe, & Hauser, 2013; Mardini, Kip, & Wilson, 2004; Mikocka-Walus, Pittet, Rossel, von Kanel, & Swiss, 2016). In studies investigating both psychological and disease predictors, psychological factors are better predictors of future disease activity than disease factors, including baseline inflammatory markers, baseline mucosal healing, baseline disease activity, disease duration, previous relapses, medications, and history of surgery (Bernstein et al., 2010; Bitton et al., 2008; Langhorst et al., 2013). Moreover, children and adolescents with IBD are at increased risk for depression, anxiety and poor quality of life (QOL), with up to 31% reporting clinically significant symptoms in our previous work (Mackner & Crandall, 2006). Worse QOL is associated with higher disease severity (Herzer, Denson, Baldassano, & Hommel, 2011; Kunz, Hommel, & Greenley, 2010). It is not yet known how stress and low QOL contribute to worsened disease activity, but indigenous gut microbes may be involved. Studies in mice show that the microbiota are necessary for stressor-induced dysregulation of the immune response, and that stressor-induced changes to the composition of the gut microbiota contribute to dysregulated colonic inflammatory responses (Mackos et al., 2016). Thus, one purpose of this study was to test the hypothesis that CD patients reporting higher levels of perceived stress would have worsened disease activity and gut microbiota compositional differences compared to those with lower levels of stress.

Microbial-produced metabolites in the intestines can impact immune system functioning (Skelly, Sato, Kearney, & Honda, 2019), and IBD is associated with significant differences

in the fecal metabolome. In both adult and pediatric IBD patients, amino acid levels are increased in the stool and serum in comparison to healthy controls (Bjerrum et al., 2015; Santoru, Piras, Murgia, Palmas, Camboni, Liggi, Ibba, Lai, Orru, Loizedda, et al., 2017). Stress exposure can also modify the gut metabolome, including robust shifts in amino acid and vitamin metabolites, which are together associated with an aberrant immune response to an *ex vivo* challenge in mice (Allen et al., Under Review). However, the relationship between stress and the gut metabolome has not been explored in a human clinical population. Therefore, an additional purpose of this study was to test the hypothesis that CD patients with high levels of perceived stress would exhibit differences in the fecal metabolome compared to those with lower stress.

2. Methods

2.1 Study Participants

Participants were patients ages 11 – 21 years who were participating in a larger, 4 year longitudinal study investigating psychosocial outcomes in adolescents with IBD. Eligibility criteria for the longitudinal study included ages 10 – 17 years and recent diagnosis of IBD. All participants in the current study had been enrolled in the longitudinal study for at least one year. For the current pilot study, 38 participants ages 11 – 21 years with Crohn's disease were asked to participate in additional study procedures investigating "how stress affects your intestines" while being scheduled for a regular longitudinal study visit. Of the 38 patients asked to participate, 27 agreed, and after informed consent, 23 submitted a stool sample. Stool and data from one participant was not included due to failing to meet study criteria for substance use (demographics are in Table 1).

2.2 Procedures

Participants in this pilot study were sent stool collection supplies and instructions in the mail to obtain a stool sample within 24 hours of the study visit. Participants were asked to store the samples in their freezers before they were transferred to a −80°C freezer at the hospital. During the longitudinal study visit, participants in this pilot study completed a Block Food Frequency Questionnaire (FFQ) and Perceived Stress Scale (PSS) in addition to the psychosocial measures for the longitudinal study. Chart reviews were conducted to obtain information about disease severity, medications and healthcare use.

2.3 Measures

2.3.1 Block Food Frequency Questionnaire—The Block Questionnaire —2005 FFQ (Block, Woods, Potosky, & Clifford, 1990) and the Block Questionnaire for Ages 8–17 — 2004 FFQ (Cullen, Watson, & Zakeri, 2008) estimate intake of a wide array of nutrients and food groups. Individual portion size is asked, and pictures are provided to enhance accuracy of quantification. Median correlations with 2-day food records are 0.8 for males and 0.7 for females (range = 0.5 – 0.9) (Maresperlman et al., 1993). FFQ data were used to calculate a Healthy Eating Index (HEI-2010) score, which is a measure of diet quality in terms of conformance with federal dietary guidance (Guenther et al., 2013).

2.3.2   Perceived Stress Scale—The PSS is a widely used measure for assessing the perception of stress in one's life (Cohen, Kamarck, & Mermelstein, 1983). Items tap how unpredictable, uncontrollable, and overloaded respondents find their lives. Internal consistency ranges from 0.84 – 0.86, and the total score is significantly correlated with number and impact of life events, depression symptoms, and physical symptoms (Cohen et al., 1983). It has been used in children as young as 11 years (Cartwright et al., 2003). For analyses of the microbial community structure, the sample was divided into high and low stress groups via median split; otherwise, the PSS total score was used as a continuous variable.

2.3.3   Short Pediatric Crohn's Disease Activity Index (sPCDAI)—The sPCDAI is a standardized, validated measure of disease activity that is derived from the longer Pediatric Crohn's Disease Activity Index (PCDAI) (Kappelman et al., 2011). It correlates well with the PCDAI, r = 0.84, and its correlation with Physician Global Assessment (PGA; r = 0.60) is similar to the correlation of the PCDAI and PGA (r = 0.61) (Kappelman et al., 2011).

2.4   Stool Sample Collection

A stool sample was collected at home using a stool collection kit that we created consisting of a commode specimen collector, stool collection tube without preservative, biohazard bag, gloves and alcohol prep pad to clean any contacted surfaces. Stool samples were collected within 24 hours of completing the PSS and FFQ. Stool samples were kept frozen until transported to the laboratory where they were stored at −80°C.

2.5   DNA isolation and preparation

Genomic DNA was isolated from stool samples using the PowerSoil® DNA Isolation Kit (MoBio) following the manufacturer's instructions. As an alternative to the recommended 250 mg of soil, approximately 250 mg of stool was added to the PowerBeads tube to undergo cell lysis. The purified DNA was eluted from the spin filter using 50 μL of solution C6 and stored at −20°C until PCR amplification.

2.6   16S rRNA gene sequencing and analysis

The 16S rRNA gene V4-V5 region was amplified using 515f and 806r primers. The 515f primer contained a DNA barcode for sample identification and samples were amplified using a 28 cycle PCR using the HotStarTaq Plus Master Mix kit (Qiagen, USA) with cycling conditions of 94°C for 3 min, followed by 28 cycles of 94°C for 30 sec, 53°C for 40 sec, and 72°C for 1 min. A final elongation step of 72°C for 5 min was also performed. Success of PCR amplification was verified using 2% agarose gel electrophoresis. Samples were purified using Ampure XP beads and pooled together in equimolar concentrations. Samples were sequenced on an Illumina MiSeq at MRDNA (Shallowater, TX, USA) following manufacturer's guidelines. Sequence data were processed using custom pipeline (MRDNA, Shallowater, TX, USA), including removal of barcodes and low quality reads (<150 bp and sequences with ambiguous base calls), denoising and chimera removal. Forward and reverse reads were joined and operational taxonomic units (OTU) were binned by clustering sequences at 3% divergence (i.e., 97% similarity). Final OTU's were taxonomically

classified using BLASTn against a curated database derived from GreenGenes/RDPII/NCBI (DeSantis et al., 2006). The QIIME pipeline was used for measures of α and β diversity.

2.7 Stool sample preparation for NMR analysis

50 mg of thawed feces were mixed with 1.0 ml of PBS (0.1M Na^+/K^+, NaN_3 30 mM, pH 7.4, 50% v/v D_2O (99.9% in D, Cambridge Isotope Laboratories, Tewksbury, MA)) containing sodium 3-trimethylsilyl [2,2,3,3-d_4] propionate (TSP-d_4) (Merck, Darmstadt, Germany) 0.0005 w/v as chemical shift internal standard. 300 mg of 0.1 mm zirconia beads were added and the mixture was vortexed for 30 s. Samples were then homogenized using a Biospec mini-beadbeater (Bartlesville, OK) for 90 s followed by two freeze thaw cycles in liquid nitrogen.

The fecal slurry was centrifuged at 11180 g for 10 mins at 4 °C and supernatants were transferred into 2 ml new EP tubes. 0.6 ml of PBS solution was added to the pellets followed by 30s of vortexing and then centrifuged at 11180 g for 10 mins at 4 °C. The supernatants were combined and centrifuged at 4°C and 16099g for 10 mins. 0.6 ml of the supernatants was transferred into 5mm NMR tubes.

2.8 NMR Spectroscopic Analysis

^{1}H NMR spectra of aqueous feces extracts were acquired at 298K on a Bruker Avance III 600 MHz spectrometer (operating at 600.06 MHz for ^{1}H and 150.90 MHz for ^{13}C) equipped with a Bruker TCI probe (Bruker Biospin, Germany). For aqueous feces extracts, a typical one-dimensional ^{1}H NMR spectrum was acquired for each sample, employing the first increment of the NOESY pulse sequence (NOESYPR1D; Relaxation delay-90-t_1-90-mixing time-90-FID) with presaturation for watert suppression. The acquisition parameters were as follows: 64 scans and 4 dummy scans, 32K data points, 90° pulse angle (11.4 us), relaxation delay 3 s, 150 ms mixing time and a spectral width of 12 ppm. The spectra were acquired without spinning the NMR tube in order to avoid artifacts, such as spinning side bands of the first or higher order. All free induction decays (FIDs) were multiplied by a decaying exponential function with a 1 Hz line broadening factor prior to Fourier transformation. The ^{1}H NMR spectra were corrected manually for phase and a polynomial fourth-order function was applied for base-line correction in order to achieve accurate and reproducible measurements upon integration of the signals of interest. Chemical shifts are reported in ppm as referenced to TSP ($\delta = 0$). To confirm NMR signal assignments, a range of 2D NMR spectra were conducted and processed for certain samples, including [1]H–[1]H correlation spectroscopy (gCOSY), [1]H–[1]H total correlation spectroscopy (gTOCSY), [1]H–[13]C edited heteronuclear single quantum correlation (gHSQC-DEPT), and [1]H–[13]C heteronuclear multiple bond correlation spectra (gHMBC). 2D NMR experiments were acquired using the 600 MHz instrument mentioned above and an 800 MHz instrument (operating at 800.13 MHz for ^{1}H and 201.21 MHz for ^{13}C) equipped with a Bruker TCI probe (Bruker Biospin, Germany) as described previously. 1D and 2D NMR spectra were processed using TopSpin 3.2 (Bruker Biospin, Germany).

2.9 Spectral Data Processing and Multivariate Data Analysis

The spectral region δ 0.50–10.0 was integrated into regions with equal width of 0.004 ppm (2.4 Hz) using the AMIX software package (V3.8, Bruker-Biospin). The region δ 4.70–4.95 was discarded due to imperfect water saturation. Prior to statistical data analysis, each bucketed region was normalized to the total sum of the spectral intensities to compensate for the overall concentration differences.

3. Data Analysis

3.1 Bacterial taxa and metabolites

A median split of PSS scores was used to create "High PSS" and "Low PSS" groups. A Student's t-test was used to compare bacterial taxa and to compare fecal metabolites between High PSS and Low PSS groups using SPSS (version 25). The Benjamini-Hochberg method (implemented in R) was used to correct for multiple comparisons.

4. Results

4.1 Perceived stress is associated with CD disease activity

Perceived stress and diet were not significantly associated, including diet measures such as HEI-2010 score, total calories, protein, carbohydrates, fat, or sugar (controlling for age), or percent of calories from protein, carbohydrates, fat, or sweets. Disease activity and perceived stress were significantly correlated, r=0.477, p=.039.

4.2 Composition differences in the gut microbiome are evident in patients with higher perceived stress

A median split of PSS scores was used to create a High PSS group (n=12) and a Low PSS group (n=10). Overall measures of microbial community composition (α- and β-diversity) were not significantly different in CD patients reporting higher levels of perceived stress vs. those reporting lower levels of perceived stress (Supplemental Figure 1). However, the relative abundances of bacteria in the phylum *Firmicutes* was significantly lower in the High PSS CD patients when compared to the Low PSS patients ($t(20) = 2.45$, $p < .05$) (Fig. 1A). Relative abundances of bacterial genera are shown in Table 2. These relative abundances were normalized by finding the arcsin of the square root of the proportion of the total sequences assigned to each genus (Galley et al., 2017). A total of 70 comparisons were made between the bacteria genera in the High vs. Low PSS groups, and after correcting for multiple comparisons, the relative abundance of two bacterial genera were found to be different in individuals reporting high levels of PSS compared to those reporting lower levels of PSS. In particular, *Anaerostipes* ($t(20) = 4.68$, p<.001, FDR $p = 0.028$) was significantly lower in the High PSS group, whereas *Parabacteroides* ($t(20) = 4.27$, $p = .001$, FDR $p = 0.035$) was significantly higher in the High PSS group (Fig. 1B). Firmicutes, Firmicutes/ Bacteroidetes ratio, *Anaerostipes*, and *Parabacteroides* abundances were not related to medication use, disease severity scores, length of disease, or diet (HEI-2010 score, total calories, protein, carbohydrates, fat, or sugar). Although there was a trend for individuals in the High PSS group to have a higher body mass index than individuals in the Low PSS group (BMI not available for 2 individuals, $t(18) = 1.93$, $p = .07$; data not shown), BMI was

also not correlated with Firmicutes, Firmicutes/Bacteroidetes ratio, *Anaerostipes*, or *Parabacteroides* abundances. Four individuals (2 in the High PSS group and 2 in the Low PSS group) were taking medication for depression. However, with this small sample size, there was no evidence that antidepressant medication affected the composition of the gut microbiome. None of the patients were taking medication for any other psychiatric illness.

4.3 Higher perceived stress is associated with an altered gut metabolome

Patients in the High PSS group also exhibited a distinct fecal metabolome as assessed with NMR. Representative superimposed ^{1}H NMR spectra of the aqueous extracts of stool samples from pediatric CD patients in the Low PSS and High PSS groups (black and red spectrum respectively), are shown in Fig. 2. As indicated by their spectra, the water extracts of feces samples contain multiple metabolites, including short chain fatty acids, amino acids, sugars, organic acids, as well as purine/pyrimidine derivatives (Kostidis et al., 2017; Lin et al., 2016). The chemical shift assignment of various metabolites was conducted on the basis of literature, the Human Metabolome Database (HMDB) and a range of 2D experiments. The use of 2D NMR was incorporated to ensure the correct chemical shift assignment of the compounds of interest. As an example, Fig. 3 is a representative HSQC-DEPT spectrum, a multiplicity edited HSQC, of a fecal aqueous extract sample, showing one bond proton–carbon correlations; positive signals (black) for the CH_3/CH carbons and negative signals (red) for the CH_2 carbons.

When the metabolome of patients in the High PSS group was compared to the metabolome of patients in the Low PSS group, four metabolites were significantly different: alanine ($t(20)=3.82$, $p<.001$), nicotinate ($t(20)=2.60$, $p<.05$), lysine ($t(20)=2.20$, $p<.05$), and phenylalanine ($t(20)=2.18$, $p<.05$) (see Table 3). However, a total of 37 statistical tests were conducted on the metabolites, and only alanine remained significantly different after correcting for multiple tests (FDR $p=.03$) (Fig. 3B). Correlation analyses were then conducted to determine whether the increased alanine was related to the observed changes *Parabacteroides* or *Anaerostipes*. Alanine was positively correlated with *Parabacteroides* ($r(20) = .501$, $p < .02$) (Fig. 3D), but was not related to *Anaerostipes* (data not shown).

5. Discussion

Consistent with our hypotheses, pediatric patients with CD who reported higher levels of stress had greater disease activity and significant differences in the composition of their intestinal microbiota when compared to patients with lower reported stress. *Anaerostipes* spp. abundance was lower, whereas *Parabacteroides* spp. abundance was higher in patients with high perceived stress. There are a number of factors that have the potential to contribute to differences in the gut microbiome. For example, diet is well known to affect microbiome composition, including Firmicutes relative abundances (David et al, 2014), which were reduced in our study. Food sensitivities, and thus, restricted diets, are common in CD patients (Prince et al., 2011; Vidarsdottir et al., 2016), but the differences in the microbiome in CD patients with higher perceived stress were not related to differences in diet. Current therapies for CD, including anti-inflammatory and immunomodulatory medications (such as corticosteroids, thiopurines, methotrexate and anti-TNF) also have the potential to affect the

microbiome. For example, the anti-TNF treatment, Infliximab, has been shown to increase α diversity and shift overall microbial community composition (Wang et al., 2018). Despite these potential effects, differences in the microbiome between CD patients with high vs. low perceived stress were independent of medication use, as well as disease severity and duration. Similarly, the gut metabolome was different in CD patients with higher perceived stress compared to patients with lower stress. The metabolites alanine, nicotinate, lysine, and phyenylalanine all differed between high- and low-perceived stress CD patients. Although only alanine remained significantly different after correcting for multiple comparisons, similar changes in nicotinate, lysine, and phenylalanine were evident in an animal model of stress (Allen et al., 2019).

There are now multiple studies comparing the microbiome in CD patients to non-CD controls, with the most consistent findings being reduced α-diversity, lower levels of bacteria in the phylum Firmicutes, and higher levels of bacteria in the phylum Gammaproteobacteria. Although we did not observe statistically significant differences in α diversity or in Gammaproteobacteria, we did observe that patients with higher perceived stress had significantly lower Firmicutes than patients with lower stress. At the genus level, *Anaerostipes* was significantly decreased in samples from the higher stress group. *Anaerostipes* is a member of the Firmicutes phylum, and the reduction in *Anaerostipes* contributed to the observed reduction in Firmicutes in high stress individuals. Interestingly, pediatric CD patients also have lower levels of *Anaerostipes* in fecal samples than do healthy controls (Wang et al., 2018).

While it is not known if more severe intestinal inflammation leads to a loss of *Anaerostipes* or if the loss of *Anaerostipes* directly contributes to disease severity, it is known that *Anaerostipes* produces the short chain fatty acid (SCFA) butyrate (Duncan, Holtrop, et al., 2004; Duncan, Louis, & Flint, 2004; Schwiertz et al., 2002). Low levels of colonic butyrate, and butyrate-producing taxa, have been identified in CD and UC (Laserna-Mendieta et al., 2018; Machiels et al., 2014). Although our NMR method was able to detect butyrate, GC-MS is a preferred method for assessing volatile compounds like SCFAs. Thus, we did not observe reductions in butyrate (or other SCFAs) in the high stress group. However, we have previously used GC-MS to show that exposing healthy mice to an experimental stressor reduces colonic SCFAs, including butyrate (Maltz et al., 2018; Maltz et al., 2019). Thus, future studies will utilize GC-MS to assess fecal butyrate as a possible mechanism linking the gut microbiome and disease severity in CD patients with higher levels of perceived stress.

In contrast to reductions in *Anaerostipes*, there were significant increases in *Parabacteroides* in CD patients with higher PSS scores. Some research has suggested that *Parabacteroides* is lower in IBD patients compared to controls and is associated with less inflammation (Zitomersky et al., 2013). This is seemingly inconsistent with our observations, but some species of *Parabacteroides* may increase inflammation. Administering *P. distasonis* to mice with existing inflammation (induced by dextran sulfate sodium (DSS)) had a significant increase in colonic inflammation (Dziarski, Park, Kashyap, Dowd, & Gupta, 2016), indicating that *Parabacteroides* spp. have the potential to exacerbate existing colonic inflammation. Moreover, mice exposed to a social stressor that enhances colonic

inflammation (Mackos et al., 2016), have increased abundances of *Parabacteroides* (Allen et al., 2019), suggesting that increases in *Parabacteroides* is a consequence of the stress response that is conserved across animal species. One limitation of this study is that the cross-sectional design does not allow for a prediction of whether these microbiome changes are a cause or a consequence of more severe intestinal inflammation in CD patients with higher perceived stress. Follow-up, longitudinal studies with multiple sampling time points are needed to determine whether changes in the abundance of *Parabacteroides* (or *Anaerostipes*) are a cause or a consequence of increased intestinal inflammation during periods of high perceived stress.

It is becoming increasingly evident that the gut metabolome (which includes small molecule metabolites produced by both the host and the resident microbiota) has far reaching effects on host health. In fact, metabolomic analyses have demonstrated that gut microbes contribute to metabolites found in blood (Wikoff et al., 2009), urine (Holmes et al., 2008; Li et al., 2008), as well as stool (Franzosa et al., 2019; Saric et al., 2008). Metabolomic profiles in stool clearly distinguish active IBD from IBD in remission and healthy controls, in part due to malabsorption that results from intestinal inflammation. This malabsorption results in higher levels of amino acids in the intestinal lumen, or stool, compared to healthy controls (Dawiskiba et al., 2014; Santoru, Piras, Murgia, Palmas, Camboni, Liggi, Ibba, Lai, Orru, Blois, et al., 2017).

In our study, fecal levels of alanine were significantly higher in individuals with higher stress. Alanine is a nonessential amino acid that can be produced by the host and/or derived from the diet and the commensal microbiota. The majority of alanine is dependent upon pyruvate, which is produced by the breakdown of glucose during glycolysis. Multiple bacteria contain enzymes that can be used to convert pyruvate into alanine (Oikawa, 2006). Thus, it is possible that the increase in fecal alanine is the result of altered bacterial metabolism, such as metabolism in *Parabacteroides* whose abundance was increased in high stress individuals and correlated with alanine levels in the stool. This hypothesis warrants further testing. However, it is equally possible that the alanine is host-derived. Increased glycolysis in skeletal muscle is a hallmark of sympathetic nervous system activation and leads to an accumulation of pyruvate. As in bacteria, mammals convert pyruvate to alanine via alanine aminotransferase. Thus, it is also a possibility that increased alanine in the stool of CD patients with higher stress is a result of increased sympathetic nervous system activity. Alternatively, the higher levels of alanine may reflect malabsorption in the small intestine; multiple studies have reported amino acid malabsorption and higher fecal amino acids (including alanine) in CD patients (Bjerrum et al., 2015; Santoru, Piras, Murgia, Palmas, Camboni, Liggi, Ibba, Lai, Orru, Blois, et al., 2017). While increased disease activity in patients with higher stress would be consistent with reduced amino acid absorption in the intestine (and thus, higher amino acids in the lumen of the intestine/feces), further studies are needed to identify mechanisms by which high stress is associated with increased fecal alanine levels.

Mice exposed to a social stressor have reductions in colonic B1, B3, and B6 vitamins (Allen et al., 2019), which led us to test whether B vitamins would also be reduced in CD patients with higher stress. Nicotinate, a B3 vitamer, was reduced in individuals with high stress.

And, when our *a priori* hypothesis that B vitamins would be reduced was tested, we found a reduction in nicotinate in individuals with higher stress. Reductions in nicotinate may have important biological functions. This B3 vitamer is the anion form of niacin, which can significantly reduce experimental colitis in mice (Salem & Wadie, 2017). Colonic bacteria are important sources of essential vitamins, including the B vitamins, and our studies in stressor-exposed mice demonstrate that stressor exposure reduces both the abundance and the diversity of bacteria capable of producing B vitamins (Allen et al., 2019). The current pilot/proof of concept study suggests that stress in human patients may also lead to reductions in intestinal B vitamins and provides the rationale for mechanism-based studies to determine whether bacterial-produced B vitamins link stressor-induced changes in the gut microbiome and disease severity in CD patients.

An important limitation of this study is that the microbiome and metabolome in patients with IBD were not compared to the microbiome and metabolome in healthy controls. Moreover, multiple comparisons were conducted to compare the microbiomes and metabolomes of CD patients with high vs. low levels of perceived stress. Because of the multiple comparisons, we used the Benjamini Hochberg method of correcting p values and maintained a conservative corrected p value of < .05 to indicate statistical significance. The benefit of this approach is that it reduces the likelihood of a Type I error (due to the multiple comparisons), but it may lead to increased Type II error. Nonetheless, we detected changes in both the microbiome and metabolome, and our results are consistent with other studies that have found that stressful periods contribute to increased severity of inflammatory diseases and conditions like IBD. Mechanisms by which the stress response enhances inflammation are not yet well understood, but murine studies indicate that gut microbes are crucial links between stress exposure and increased intestinal inflammation. This led us to assess whether there is any evidence of disrupted microbiota in IBD patients with higher levels of perceived stress compared to patients with lower levels of perceived stress. This pilot study suggests that the gut microbiome and metabolome differ in CD patients with higher perceived stress. However, additional longitudinal studies are needed to determine whether the changes in the microbiome and metabolome contribute to intestinal inflammation, or are the result of increased disease activity during stressful periods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Higher levels of perceived stress were associated with worse disease activity

- Lower relative abundances of bacteria in phylum Firmicutes in higher stress group

- Genus *Anaerostipes* was lower and *Parabacteroides* was higher in the high stress group

- Differences were independent of medications, disease severity, disease duration, diet

- Levels of the metabolite alanine were higher in youth with higher stress
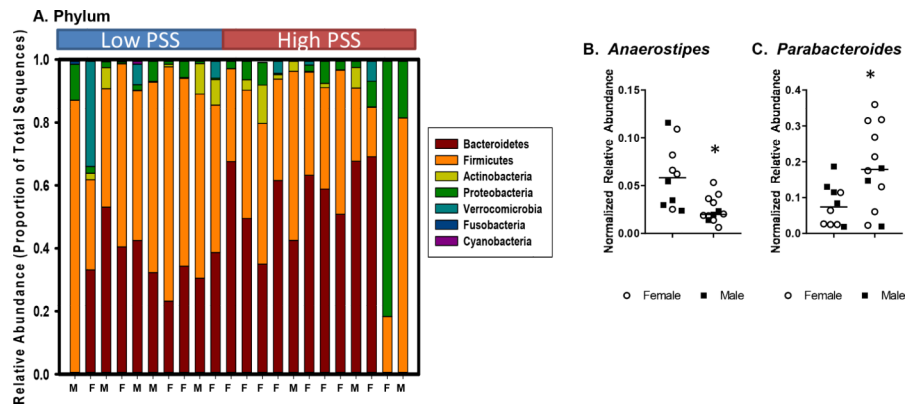
Figure 1: Crohn's disease patients with higher perceived stress have differences in specific bacterial taxa compared to patients with lower perceived stress.

A) Crohn's disease patients in the High PSS group had significantly lower relative abundances of Firmicutes (as determined by percentage of total sequences) (p<.05). B) Crohn's disease patients in the High PSS group had a significant decrease in the relative abundance of *Anaerostipes* and a significant increase in *Parabacteroides* (FDR p<.05). Data show the arcsin of the square root of the proportion for each bacterial genus.
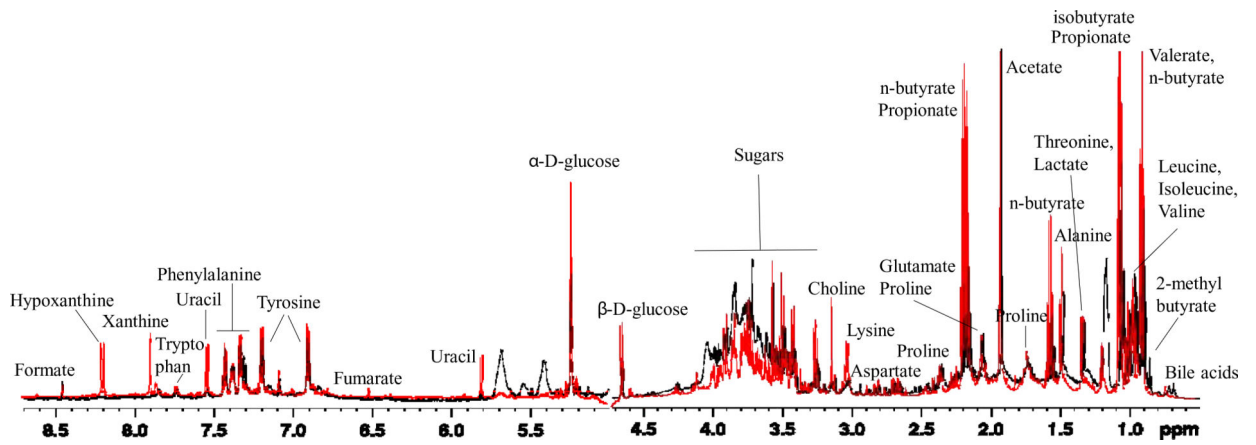
Figure 2: NMR spectra differ in CD patients with higher vs. lower levels of perceived stress.
Superimposed 1D 1H NMR spectra of the aqueous extracts of stool samples from pediatric CD patients in the Low PSS and High PSS groups (black and red spectrum respectively).
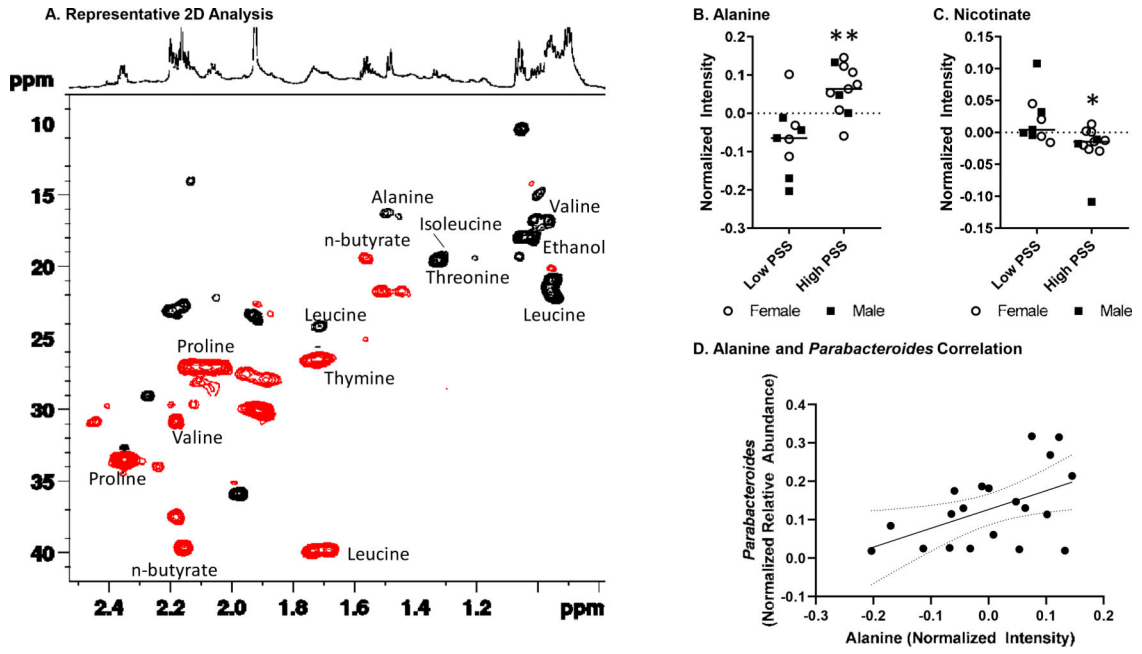
Figure 3: Fecal metabolites differ in CD patients with higher vs lower perceived stress.
A) Multiplicity edited HSQC of a stool aqueous extract sample, displaying one bond ^1H-^{13}C correlations; positive signals (black) for the CH_3/CH carbons and negative signals (red) for the CH_2 carbons. B) Relative intensities of alanine and C) nicotinate are significant different in CD patients in the High PSS group. D) Normalized relative abundance of *Parabacteroides* was significantly correlated with alanine intensity ($p < .05$).

** p and FDR<.05. *p<.05 prior to FDR correction; FDR p=.13.

Table 1.

Patient demographics, disease severity, and medication usage.

	n=22
Mean age (yrs; s.d.)	17.00 (2.75)
Min-max	11.58–21.00
% Male	36%
% Caucasian	67%
% Married (parents)	73%
Median family income	$65,000
Disease duration (yrs; s.d.)	3.68 (1.60)
Min-max	1.17–5.92
Disease severity[a]	
Remission	68%
Mild	21%
Moderate/severe	11%
Current corticosteroid	0%
Current biologic	57%
Current antidepressant	18%
Clinic visits past 6 mo. (median) (Min-max)	2.00 (0–4)
Hospitalized past 6 mo.	5%
Surgery past 6 mo.	0%

[a]Short Pediatric Crohn's Disease Activity Index

Table 2:

Fecal microbes in Crohn's disease patients with Low or High PSS Scores

		Low PSS				High PSS		
Anaerostipes **		0.060	+/−	0.011		0.025	+/−	0.004
Parabacteroides **		0.079	+/−	0.018		0.184	+/−	0.033
Anaerotruncus		0.019	+/−	0.004		0.009	+/−	0.002
Blautia		0.331	+/−	0.037		0.263	+/−	0.042
Eggerthella		0.015	+/−	0.004		0.008	+/−	0.001
Eisenbergiella		0.017	+/−	0.005		0.013	+/−	0.003
Tyzzerella		0.052	+/−	0.018		0.016	+/−	0.002
Ruminoclostridium		0.090	+/−	0.025		0.041	+/−	0.010
Shigella		0.088	+/−	0.020		0.049	+/−	0.010
Parasutterella		0.047	+/−	0.017		0.069	+/−	0.025
Clostridium		0.294	+/−	0.034		0.218	+/−	0.032
Subdoligranulum		0.101	+/−	0.020		0.067	+/−	0.016
Fusobacterium		0.024	+/−	0.013		0.005	+/−	0.001
Erysipelatoclostridium		0.019	+/−	0.005		0.011	+/−	0.002
Barnesiella		0.019	+/−	0.010		0.019	+/−	0.012
Faecalitalea		0.015	+/−	0.008		0.008	+/−	0.002
Oscillospira		0.055	+/−	0.015		0.040	+/−	0.014
Turicibacter		0.019	+/−	0.005		0.036	+/−	0.009
Eubacterium		0.180	+/−	0.034		0.213	+/−	0.038
Intestimonas		0.014	+/−	0.005		0.007	+/−	0.002
Butyricimonas		0.005	+/−	0.000		0.028	+/−	0.018
Lactococcus		0.005	+/−	0.001		0.007	+/−	0.002
Akkermansia		0.134	+/−	0.063		0.064	+/−	0.025
Lachnoclostridium		0.115	+/−	0.025		0.120	+/−	0.027
Desulfovibrio		0.003	+/−	0.001		0.015	+/−	0.007
Lachnobacterium		0.021	+/−	0.015		0.009	+/−	0.002
Sporobacter		0.016	+/−	0.007		0.008	+/−	0.003
Megasphaera		0.093	+/−	0.070		0.048	+/−	0.023
Roseburia		0.058	+/−	0.014		0.080	+/−	0.024
Fusicatenibacter		0.091	+/−	0.019		0.056	+/−	0.011
Lactobacillus		0.037	+/−	0.011		0.019	+/−	0.004
Anaerovorax		0.008	+/−	0.002		0.009	+/−	0.002
Parasporobacterium		0.021	+/−	0.010		0.009	+/−	0.003
Cellulosilyticum		0.005	+/−	0.002		0.018	+/−	0.013
Sutterella		0.020	+/−	0.004		0.096	+/−	0.039
Collinsella		0.027	+/−	0.010		0.011	+/−	0.004

		Low PSS			High PSS	
Entercococcus	0.012	+/−	0.001	0.046	+/−	0.031
Intestinibacter	0.079	+/−	0.029	0.056	+/−	0.015
Alistipes	0.120	+/−	0.041	0.155	+/−	0.034
Dialister	0.085	+/−	0.037	0.052	+/−	0.014
Klebsiella	0.006	+/−	0.001	0.009	+/−	0.003
Bacteroides	0.520	+/−	0.057	0.616	+/−	0.085
Lachnospira	0.019	+/−	0.007	0.013	+/−	0.005
Sporobacterium	0.003	+/−	0.001	0.003	+/−	0.001
Prevotella	0.049	+/−	0.032	0.052	+/−	0.029
Pantoea	0.031	+/−	0.006	0.124	+/−	0.092
Acidominococcus	0.019	+/−	0.010	0.038	+/−	0.029
Haemophilus	0.067	+/−	0.032	0.041	+/−	0.014
Coprococcus	0.036	+/−	0.014	0.026	+/−	0.007
Flavonifactor	0.031	+/−	0.006	0.039	+/−	0.010
Faecilbacterium	0.269	+/−	0.070	0.136	+/−	0.035
Actinomyces	0.011	+/−	0.001	0.007	+/−	0.001
Phascolarctoba	0.036	+/−	0.012	0.054	+/−	0.021
Allisonella	0.003	+/−	0.001	0.008	+/−	0.006
Porphyromonas	0.005	+/−	0.001	0.004	+/−	0.001
Odoribacter	0.033	+/−	0.010	0.037	+/−	0.011
Lactonifactor	0.007	+/−	0.002	0.007	+/−	0.002
Veillonella	0.032	+/−	0.012	0.044	+/−	0.012
Ruminococcus	0.119	+/−	0.023	0.091	+/−	0.024
Hespellia	0.004	+/−	0.002	0.004	+/−	0.001
Rothia	0.003	+/−	0.001	0.003	+/−	0.001
Bilophila	0.024	+/−	0.008	0.032	+/−	0.008
Gemella	0.003	+/−	0.001	0.003	+/−	0.001
Dorea	0.072	+/−	0.011	0.063	+/−	0.012
Streptococcus	0.038	+/−	0.005	0.031	+/−	0.005
Bifidobacterium	0.118	+/−	0.038	0.112	+/−	0.033
Sporoanaerobacterium	0.013	+/−	0.003	0.009	+/−	0.003
Anaerospora	0.010	+/−	0.004	0.007	+/−	0.002
Paludibacter	0.053	+/−	0.021	0.030	+/−	0.015
Adlercreutzia	0.006	+/−	0.002	0.005	+/−	0.002
	4.074			3.833		

Data are the normalized relative abundances calculated by the arcsin of the square root of the sequence proportions assigned to each genus.

[**]
FDR corrected p < .05

Table 3:

Fecal metabolites in CD patients with Low or High PSS scores.

		Low PSS			High PSS		
Alanine [**]		−0.067	+/−	0.030	0.063	+/−	0.019
Nicotinate [*]		0.020	+/−	0.013	−0.020	+/−	0.010
Lysine [*]		−0.047	+/−	0.036	0.043	+/−	0.017
Phenylalanine [*]		−0.093	+/−	0.039	0.030	+/−	0.039
Tyrosine		−0.056	+/−	0.026	0.031	+/−	0.033
Uracil		−0.019	+/−	0.009	0.018	+/−	0.015
Fumerate		0.004	+/−	0.004	−0.007	+/−	0.004
Adenosine		0.026	+/−	0.020	−0.015	+/−	0.017
Bile Acids		0.059	+/−	0.030	−0.004	+/−	0.026
Uroconate		−0.014	+/−	0.007	0.020	+/−	0.020
Xanthine		−0.007	+/−	0.008	0.010	+/−	0.011
UDP glucoronate		0.039	+/−	0.051	−0.032	+/−	0.040
Glutamine		0.032	+/−	0.042	−0.027	+/−	0.035
Hypoxanthine		−0.010	+/−	0.015	0.013	+/−	0.015
Adenosine		0.010	+/−	0.010	−0.013	+/−	0.017
2-methylbutyrate		0.012	+/−	0.018	−0.010	+/−	0.017
n-butyrate		0.054	+/−	0.064	−0.025	+/−	0.066
Iso-caproate		0.000	+/−	0.018	0.022	+/−	0.017
Asparagine		0.058	+/−	0.056	−0.010	+/−	0.057
Methionine		0.006	+/−	0.006	−0.001	+/−	0.006
Galactose		0.026	+/−	0.030	−0.002	+/−	0.019
Glycine		−0.027	+/−	0.017	−0.006	+/−	0.023
3-hydroxyphenylacetate		−0.042	+/−	0.004	−0.034	+/−	0.011
Tryptophan		−0.025	+/−	0.005	−0.014	+/−	0.015
Methylamine		0.009	+/−	0.012	−0.001	+/−	0.011
Ribose		−0.026	+/−	0.032	−0.009	+/−	0.017
p-Cresol		−0.004	+/−	0.017	−0.018	+/−	0.029
Histidine		−0.003	+/−	0.008	−0.006	+/−	0.006
5-aminopentanoate		0.006	+/−	0.004	0.003	+/−	0.007
Succinate		0.020	+/−	0.081	−0.009	+/−	0.057
Beta-D-glucose		0.033	+/−	0.061	0.016	+/−	0.028
Formate		0.002	+/−	0.005	−0.001	+/−	0.011
Alpha-D-glucose		0.001	+/−	0.030	−0.005	+/−	0.018
Fructose, lactate		0.015	+/−	0.048	0.022	+/−	0.055
Aspartate		0.027	+/−	0.120	0.012	+/−	0.125

[*]
p < .05 prior to FDR correction.

**
FDR corrected p < .05.

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