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# Establishing a relationship between bacteria in the human gut and Complex Regional Pain Syndrome

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ABSTRACT

Complex Regional Pain Syndrome (CRPS) is a serious and painful condition involving the peripheral and central nervous systems. Full comprehension of the disorder's pathophysiology remains incomplete, but research implicates the immune system as a contributor to chronic pain. Because of the impact gastrointestinal bacteria have in the development and behavior of the immune system, this study compares the GI microbial communities of 16 participants with CRPS (5 of whom have intestinal discomforts) and 16 healthy controls using 454 sequencing technology. CRPS subjects were found to have significantly less diversity than their healthy counterparts. Statistical analysis of the phylogenetic classifications revealed significantly increased levels of Proteobacteria and decreased levels of Firmicutes in CRPS subjects. Clustering analysis showed significant separation between healthy controls and CRPS subjects. These results support the hypothesis that the GI microbial communities of CRPS participants differ from those of their healthy counterparts. These variations may hold the key to understanding how CRPS develops and provide information that could yield a potential treatment.



## 1. Introduction

### 1.1. Complex Regional Pain Syndrome

Complex Regional Pain Syndrome (CRPS) is a serious and painful condition that generally develops after an inciting event such as injury, illness or surgery and cannot be explained by another medical diagnosis. The array of symptoms that accompany this condition can be classified into four main categories: abnormal pain processing, vasomotor changes, trophic changes, and impaired motor functions (Alexander et al., 2007; Schwartzman et al., 2009; Harden et al., 2010). Each category contains multiple indications which a person may exhibit individually or in concert with other symptoms. While those with CRPS present similar categorical traits, the arrangement and degree to which these symptoms are expressed vary across the population resulting in the creation of disease subsets (Alexander et al., 2012). Additionally, there is no one specific therapy that wholly addresses the effects of this varied condition (Goebel et al., 2010; Alexander et al., 2012). It is estimated that there are between 200,000 and 1.2 million Americans with CRPS and in a recent study, 81% of those affected by CRPS reported pain levels that precluded them from remaining in the workforce (Schwartzman et al., 2009; Mailis, 2003). There is evidence that early recognition and treatment of CRPS increases the chance of recovery though current treatments are largely ineffective (Goebel et al., 2010; Watkins et al., 2007). Although the mechanisms behind CRPS have not been fully identified, research has shown that neurogenic inflammation plays a significant role and appears to be modulated by both the central nervous and immune systems (Marchand et al., 2005; Alexander et al., 2007; Schwartzman et al., 2011; Goebel et al., 2010).

### 1.2. Gut-brain axis

The gut-brain axis is a bi-directional communication network involving the sympathetic, parasympatheic, and enteric systems (Collins and Bercik, 2009; Cryan and O'Mahony, 2011; Mayer and Tillisch, 2011; Bercik et al., 2012). There is a perpetual flow of information detailing bodily performance that is acquired by the brain and used to maintain homeostatic levels – among them the digestive and "gut-associated immune" systems (Mayer and Tillisch, 2011). Sensory signals are carried by the enteric, spinal,

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and vagal pathways. Of particular note is the vagus nerve which is in contact with processes of dendritic cells that extend through the epithelial layer of the GI tract (Collins and Bercik, 2009; MacDonald and Monteleone, 2005; Round and Mazmanian, 2009). This nerve carries signals to the CNS and is essential in regulating emotion, pain, and immune response, it has been named in the immune-to-brain pathway, and has been associated in the development of sickness responses (MacDonald and Monteleone, 2005; Watkins and Maier, 2005; Collins and Bercik, 2009; Round and Mazmanian, 2009; Mayer and Tillisch, 2011). Molecules involved in the transmission of information include cytokines, hormones, endotoxins, and neuropeptides (Watkins and Maier, 2005; Bercik et al., 2012).

Under healthy circumstances, interoceptive information is not generally perceived consciously. However in persons experiencing functional abdominal pain syndrome, they are keenly aware of this transmission and experience extended pain/discomfort (Mayer and Tillisch, 2011). It has been suggested that these pain states result from dysregulated interactions between the gut lumen and mucosa, the enteric nervous system, and the central nervous system – all culminating in modification of affect, perception, GI motility, and in certain conditions, immune function (Mayer and Tillisch, 2011).

### 1.3. Importance of bacteria

The GI bacterial community is purported to contain on the order of $10^{13}$–$10^{14}$ organisms, harbors approximately 1000 different species, and is considered to have the most diversity among other human-host environments such as the skin and oral cavity (Tancrede, 1992; Hugenholtz and Tyson, 2008; Savage, 1977; Quince et al., 2009; Reeder and Knight, 2010; Van den Abbeele et al., 2011). Bacteria within the gut are vital to nutrient breakdown and absorption; they prevent colonization of pathogens, can metabolize toxins on a scale equal to that of the human liver and provide a structural backbone to a functional immune system (Collado et al., 2009; Kurokawa et al., 2007; MacDonald and Monteleone, 2005; Round and Mazmanian, 2009). Along the GI tract, dendritic cells are directly stimulated by contact with microbes, prompting the body to develop an immune response which includes the release of cytokines (Collins and Bercik, 2009; MacDonald and Monteleone, 2005; Round and Mazmanian, 2009).

The importance of gastrointestinal (GI) bacteria and their ability to influence human health has been the focus of many human microbiome studies. To date, many of these studies have attempted to ascertain the constituents of a healthy GI microbial community or to describe community differences in the face of diet differences, obesity, antibiotic usage, colon cancer, or inflammatory bowel diseases (IBDs) (Eckburg et al., 2005; Ley et al., 2006; Manichanh et al., 2006; Dethlefsen et al., 2008; Sobhani et al., 2011). While there is still some debate as to what constitutes the 'core' GI microbial community, the prevailing belief is that a GI system housing an unbalanced microbial community leads to health issues involving inflammation (Round and Mazmanian, 2009). It seems intuitive that GI bacteria can be correlated to GI maladies, however there are a number of studies that have found a link between GI bacteria and behavior, stress, depression, sickness response, and pain perception (Collins and Bercik, 2009; Forsythe et al., 2010; Bravo et al., 2011; Finegold et al., 2010; Watkins and Maier, 2005; Quan and Banks, 2007). In particular, Amaral demonstrated that GI bacteria could influence how rodents perceived pain that was located at their extremities (Amaral et al., 2008).

### 1.4. Targeting bacteria

In order to identify the bacterial communities of each sample, this study targeted the 16S rRNA (small subunit ribosomal RNA) gene. The gene is comprised of approximately 1600 nucleotides, with conserved and variable regions (Petrosino et al., 2009). The conserved areas allow researchers to design primers that will recognize all bacteria while the areas of variability provide valuable phylogenetic information (Hamady and Knight, 2009; Petrosino et al., 2009). For this investigation, bacterial sequences were procured with 454 sequencing technology using primers directed at the V2 conserved region of the 16S rRNA gene.

## 2. Methods

### 2.1. Subjects

The study was undertaken at Drexel University College of Medicine (DUCOM) in Philadelphia PA and was approved by the Internal Review Board (IRB). Sixteen CRPS subjects and 16 healthy controls were enrolled in this study. Informed consent was obtained from all subjects prior to their participation. As CRPS is approximately four times more prevalent in women than men, only women were recruited for this investigation (Schwartzman et al., 2009; de Mos et al., 2007). Subjects with CRPS (CRPS_All $n = 16$) were recruited from the DUCOM Pain Clinic and met the International Association for the Study of Pain (IASP) diagnostic criterion for CRPS (Harden et al., 2010). Of the 16 CRPS subjects, 14 of them were classified as having Type I CRPS. A subset of the CRPS population (CRPS_GI $n = 5$) experienced gastrointestinal discomforts (e.g. pain, constipation, diarrhea) but did not have a diagnosis of IBD or other GI disorders. Participation was offered to patients using the following inclusion criteria: (1) female; (2) 20–55 years of age; (3) physician diagnosis of CRPS I (no demonstrable nerve lesion) or II (identifiable nerve lesion); (4) willingness to complete a self-administered written survey; (5) a willingness to provide a fecal sample; (6) reside within the Philadelphia region; and (7) exercise non-vegetarian eating habits. Exclusion criteria included: (1) the inability to complete the questionnaire; (2) other serious medical conditions; (3) use of antibiotics, narcotics or colon cleansing within 3 months prior to sample collection; and (4) women who had hysterectomies, were pregnant or were on hormone replacement regiments. The control subjects ($n = 16$) consisted of healthy females with no medical conditions and with the exception of having a pain diagnosis, adhered to same participation requirements as the CRPS population. In addition to collecting a fecal sample, all participants completed a standardized questionnaire constructed for this study that captured self-reported demographic information, a history of medical diagnoses (including age of onset of their symptoms and age of their diagnosis by a physician), medication and/or nutritional supplement usage, and a synopsis of their typical food and drinking habits.

### 2.2. Sample collection and transport, DNA extraction and sequencing

In order to assure our study was comparable to other studies, this study followed the protocol employed by the Human Microbiome Project (Peterson et al., 2009; Gevers et al., 2012). Fecal samples were collected by study participants in their homes. Collected samples were placed in a stool specimen container and immediately placed under anaerobic conditions, stored in a cooler at −4 °C and transported at 4 °C to the laboratory within 24 hours. Once in the laboratory the samples were weighed, partitioned into shipping and storage tubes and stored in a freezer at −80 °C. Analysis samples were shipped overnight express with dry ice to the Research Testing Laboratories (Lubbock, TX) for sequencing. Methods for DNA extraction, amplification, and 454 sequencing follow those referenced in a recent publication by Finegold et al. (2010).

### 2.3. Separation of CRPS into sub-groups

Because it is known that people with GI maladies exhibit distinct microbial communities, the CRPS group (CRPS_All) was often split into two sub-groups; those with GI issues (CRPS_GI) and those without GI issues (CRPS_NOGI). The reasoning behind this approach was to ensure that those with GI complications were not the driving force behind any differences found between the CRPS_All group and the Control group. Significance was determined using one-way ANOVA.

### 2.4. Sequence processing pipeline and microbial analysis

The microbial analysis was performed using command-line versions of Qiime (Quantitative Insights Into Microbial Ecology) and RDP (Ribosomal Database Project) (Caporaso et al., 2010b; Wang et al., 2007; Cole et al., 2009). Qiime consists of its own, as well as numerous third-party tools, designed to compare and analyze microbial communities (Parameswaran et al., 2007; Edgar, 2010; Li and Godzik, 2006; Haas et al., 2011; DeSantis et al., 2006). Within this frame-set, users are free to tailor their analysis by selecting which tools are used and by tuning the tool's parameters. Qiime incorporates the RDP classifier but not RDP's library comparison tool. The 454 sequences were processed and analyzed in an Unix-environment.

Prior to analyzing the dataset, sequences were inspected to ensure proper labeling (barcoding) (Parameswaran et al., 2007). After the mapping check, the dataset was filtered to remove sequences <250 nucleotides or >850 nucleotides in length. Reads with poor quality scores (<25), ambiguous bases, mutations in primers and barcodes, or reads containing more than 6 homopolymers (e.g. six or more repeats of a particular nucleotide) were also excluded from further analysis.

Sequences were subsequently sorted in order of decreasing length and clustered at 97% identity to establish the operational taxonomic units (OTUs) using Uclust and Cd-hit (Edgar, 2010; Li and Godzik, 2006). Although there are known discrepancies, sequences grouped together at 3% divergence are generally accepted as belonging to the same species during taxonomic classification (Petrosino et al., 2009).

Representatives from each OTU were aligned with PyNAST using the Green Genes pre-aligned core set as a template and a Lane mask (DeSantis et al., 2006; Lane, 1991). ChimeraSlayer was run on the aligned sequences to identify and remove any sequences generated by PCR error (i.e. chimera) (Haas et al., 2011).

Two non-parametric estimators were used to investigate the richness and diversity of a sample. The Chao1 Estimator and the Shannon Index are common ecological tools employed to look at a population's variation and diversity (Shannon, 1948; Chao et al., 2000). The Shannon Index uses the abundance of each species to determine the sample's diversity while the Choa1 Estimator uses the number of OTUs, the number of OTUs containing only one sequence, and the number of OTUs with only two sequences to assess the sample's richness.

Rarefaction curves which are graphical representations of diversity were generated by randomly selecting a particular number of sequences from a group's sample set and determining the number of species (OTU's) found within that set. This process was repeated 100 times (with replacement) and the resulting average values were used to create a rarefaction plot.

OTUs were assigned to a taxonomy with the RDP Classifier at $\geqslant$80% bootstrap support (Caporaso et al., 2010a; DeSantis et al., 2006; Wang et al., 2007). After taxonomic classification, the cohort of samples belonging to the same group (e.g. CRPS, Control) were combined into a single library. Library compositions were compared between the Control and CRPS groups using RDP's Command-line Library comparison tool. This tool calculates the probability of the observed frequency differences between libraries given equal frequencies within the two studied libraries.

In order to visualize possible sample separation, Principle Coordinate Analysis (PCoA) using an unweighted UniFrac distance matrix was employed (Lozupone and Knight, 2005; Ramette, 2007). PCoA belongs to a set of clustering methods which uses a distance matrix to describe all the samples within the dataset. This matrix is used to calculate the principle dimensions which depict the differences between members from separate categories (e.g. study groups). PCoA organizes the dimensions to be those that best discriminate between the specified categories and not just between individual samples.

### 2.5. Statistical analyses

Statistical analyses were performed using the statistical package SPSS (version 19) (SPSS, 2010). When computing the differences between two groups, the Student's t-test was used. To assess significant differences between more than 2 groups, analysis of variance (ANOVA) was used. In order to determine if a particular factor (e.g. disease state, age, BMI, medication) had a significant association with the PCoA axes, multivariate analysis of variance (MANOVA) was performed on the first three PCoA tables (Table 2) using Pillai's criterion. Univariate ANOVA was run on any significant MANOVA results to show which axis was influential in the PCoA plots (Table 2). For all types of analysis, significance was defined at $p \leqslant 0.05$.

## 3. Results

### 3.1. Subject demographics

A total of 32 subjects (16 CRPS (5 with intestinal discomforts), 16 controls) were enrolled in this study. All CRPS subjects were recruited from the DUCOM pain clinic and met the IASP criteria for CRPS. Clinical examination revealed irregular pain processing in all subjects; the most pronounced symptom was mechano allodynia. Of the 16 subjects, 14 were classified as having Type I CRPS. All participants were white females, between the ages of 23–51 residing in the Philadelphia region. The most common medications taken by the CRPS study subjects were anti-epileptics (63%), anti-depressants (57%), and anti-anxiolytics (31%). The number of subjects in each group, their age, body mass index, and their pain levels and disease duration (when applicable) can be found in Table 1. Using the Student's t-test, no significant differences in BMI or age ($df = 30$, $p > 0.05$) were found.

### 3.2. Sequence processing

At total of 180,896 sequences were returned from Research Testing Laboratories. Post filtering, the dataset consisted of 96,224 sequences with an average number of sequences per participant at 3007 (range 1484–9767). After chimera removal, the dataset consisted of 88,958 sequences. On average, the number of sequences per participant was 2505 (range 1308–9157) (Table 1).

### 3.3. Sample diversity

The Choa1 Estimator and Shannon Index were the two non-parametric estimators used to investigate the richness and diversity of each sample. Information about the number of sequences, OTUs, the diversity assessment, and their mean values are in Table 1. The mean values for OTUs, Chao1 Estimators, and Shannon Indices were lower in all CRPS groups when compared to the

**Table 1**
Characteristics of study participants (N) in each group at time of sampling. The mean and range of age and disease duration are given in years. The mean ± the standard error is given for the body mass index (BMI). The pain score is on a scale between 1–10. Mean values for number of sequences, OTUs (grouped at 97% (species level)), richness estimator (Chao1) and diversity (Shannon) index for each group are also listed. Mean values for Chao1 Estimator and Shannon Index are accompanied by the ± standard error of their means. Assuming equal proportions of each OTU, the maximum possible Shannon Index is 8.23.

| | CRPS_NOGI | CRPS_GI | CRPS_ALL | Controls |
|---|---|---|---|---|
| Group size | 11 | 5 | 16 | 16 |
| Age years (range) | 40.45 (23–51) | 37.20 (31–49) | 39.44 (23-51) | 35.63 (24–49) |
| BMI | 25.70 ± 1.65 | 24.10 ± 1.37 | 25.19 ± 1.21 | 23.68 ± 0.70 |
| Disease duration (range) | 4.82 (1–12) | 7.00 (1–11) | 5.50 (1–12) | NA |
| Pain level (range) | 5.2 (3–8) | 5.9 (3–8) | 5.7 (3–8) | NA |
| Sequences (range) | 2819.27 (1853–4787) | 1857.20 (1581–2235) | 2519.25 (1581–4787) | 3040.63 (1308–9157) |
| OTUs (range) | 280.45 (195–392) | 203.20 (116–272) | 256.31 (116–392) | 328.63 (145–591) |
| Chao1 estimator | 520.76 ± 44.18 | 400.27 ± 40.06 | 483.10 ± 35.16 | 651.75 ± 54.12 |
| Shannon index | 3.89 ± 0.15 | 3.65 ± 0.19 | 3.82 ± 0.12 | 4.12 ± 0.12 |

**Table 2**
*P-value*s from MANOVA (a) and ANOVA (b) analyses on PCoA axes. Analysis incorporated an UniFrac distance matrix and environmental characteristics of the sample's subject. Bolded values indicate significant *P*-values.

| Source of variation | Pillai's trace | Hypothesis d.f. | Error d.f. | *F* | *P* |
|---|---|---|---|---|---|
| (a) *Multivariate analysis* | | | | | |
| CRPS_NOGI, CRPS_GI & Controls | 0.189 | 6 | 56 | 4.401 | **0.001** |
| CRPS_All & Controls | 0.508 | 3 | 28 | 9.647 | **0.000** |
| Age | 0.251 | 9 | 84 | 0.852 | 0.571 |
| BMI | 0.149 | 6 | 56 | 0.751 | 0.611 |
| | Type III SS | d.f. | *F* | *P* | |
| (b) *Univariate analysis* | | | | | |
| CRPS_NOGI, CRPS_GI & Controls | | | | | |
| PCoA1 | 0.402 | 2 | 18.298 | **0.000** | |
| PCoA2 | 0.012 | 2 | 0.313 | 0.734 | |
| PCoA3 | 0.27 | 2 | 0.956 | 0.396 | |
| CRPS_All & Controls | | | | | |
| PCoA1 | 0.296 | 1 | 23.76 | **0.000** | |
| PCoA2 | 0.018 | 1 | 0.738 | 0.401 | |
| PCoA3 | 0.002 | 1 | 0.122 | 0.731 | |

Control group. T-test results between the CRPS_All and the Control group yielded significant differences in both the number of species (OTUs) ($df = 30$, $p \leqslant 0.05$) and the Chao1 Estimator ($df = 30$, $p \leqslant 0.05$). Near significance ($df = 30$, $p < 0.1$) can be seen in the Shannon (diversity) Index.

Interestingly, when we divided the CRPS subjects into two groups; those with intestinal difficulties (CRPS_GI) and those without (CRPS_NOGI), diversity measures remained lower than the controls while maintaining no significant differences between the two CRPS sub-populations.

Rarefaction curves assembled from combined datasets can be seen in Fig. 1. The curves indicate that as the number of sampled sequences increases, the Control group consistently scored the highest number of species. Within the CRPS_All group, the CRPS_NOGI group contains a greater species richness than the CRPS_GI group. That each curve continues to increase and has not begun to plateau suggests that not all species have been discovered and more reads should be obtained during the sequencing process (Heck et al., 1975). However, this should not obviate the result of higher species occurrence reported in the Control group.

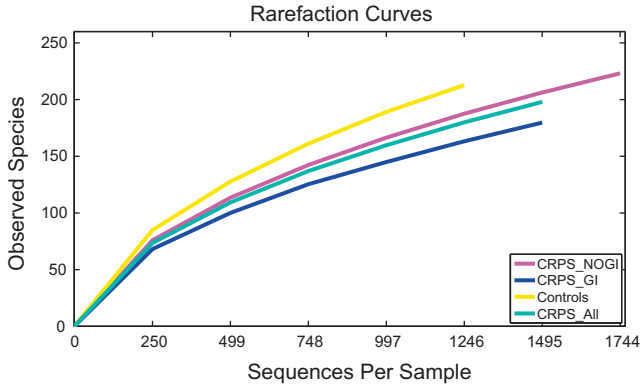


**Fig. 1.** Rarefaction analysis of V2-targeted pyrosequenced reads of the bacterial 16S rRNA gene recovered from human fecal specimens. The rarefaction curves were constructed at 97% (species level) sequence similarity and were pooled together according to disease group specification.

### 3.4. Compositional classification and comparison

A breakdown of each study group at the phylum level can be seen in Fig. 2. The Control group had the highest proportion of the Firmicutes phylum at 64.8% (CRPS_All 46.21%, CRPS_GI group 51%, CRPS_NOGI group 44%). Variation in the proportion of Proteobacteria between the two groups was also pronounced. Proteobacteria contribution percentages were 0.078% for the Control group and 7.02% for the CRPS_All group (CRPS_GI 11.3%, CRPS_NOGI group 5.1%). T-test p-values were found to be significant for Firmicutes and Proteobacteria phyla ($df = 18.97$, $p \leqslant 0.05$ and $df = 15.68$, $p \leqslant 0.05$ respectively, equal variance not assumed). These results indicate a structural difference in the make up of the bacterial communities between the Control and all CRPS groups. In order to determine if this conclusion was well founded, RDP's Command-line Library comparison tool was used (Cole et al., 2009). This tool calculates the probability of seeing the occurrence rate of a given taxon in one library appearing at the same frequency in a second

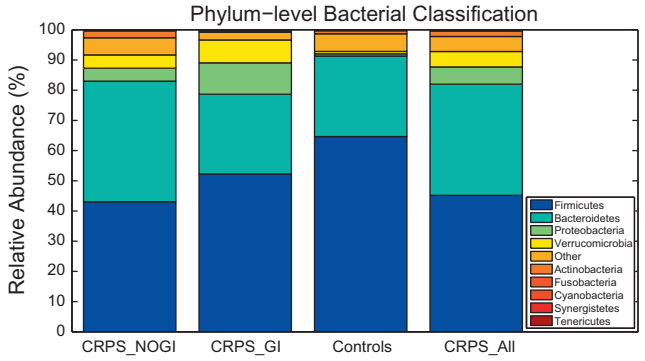


**Fig. 2.** The relative abundance of the bacterial composition, seen at the phylum level. Participants with CRPS show a reduction in Firmicutes and increased levels of Proteobacteria. The CRPS_GI ($n = 5$) contains CRPS subjects that expressed GI issues, the CRPS_NOGI are comprised of CRPS subjects sans GI complications, the CRPS_All group is a cohort of all the CRPS subjects. Verrucomicrobia appears elevated in the CRPS groups, however the Student's t-test did not produce significant results. Further inspection revealed the phylum's increased levels can be attributed to a few CRPS subjects.

library. When compared to the Control group, CRPS groups were found to have significantly distinct representations of Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria, Synergistetes, and Verrucomicrobia phyla ($p \ll 0.01$).

### 3.5. Bacterial community structure comparisons

In order to visually inspect how samples clustered together, PCoA (unweighted UniFrac matrix) was implemented. When samples where assessed by other patient characteristics (e.g. age, BMI, disease duration, pain status, medication), the only identifiable delineation occurred when samples were analyzed according to disease state (e.g. CRPS, control) (Fig. 3). In Fig. 3, the Control group clusters more closely than CRPS subjects, indicating a similarity in the group's microbial structure. P1 vs P3 captures a slight clustering of CRPS_GI subjects, demonstrating that there may be something distinct about this sub-population.

## 4. Discussion

Although the pathophysiology of CRPS is not completely understood, it is thought to include a maladaptive response to nervous system damage involving immune and inflammatory pathways (Costigan et al., 2009; Watkins and Maier, 2005). Converging evidence identifies the role of the immune system as a contributor to the development and continuation of chronic pain (Costigan et al., 2009; Watkins and Maier, 2005). As GI bacteria have a profound effect on the functioning of the immune system, this study investigated whether differences existed in the bacterial communities in healthy controls and CRPS sufferers. There was no significant difference in age or BMI between the study groups.

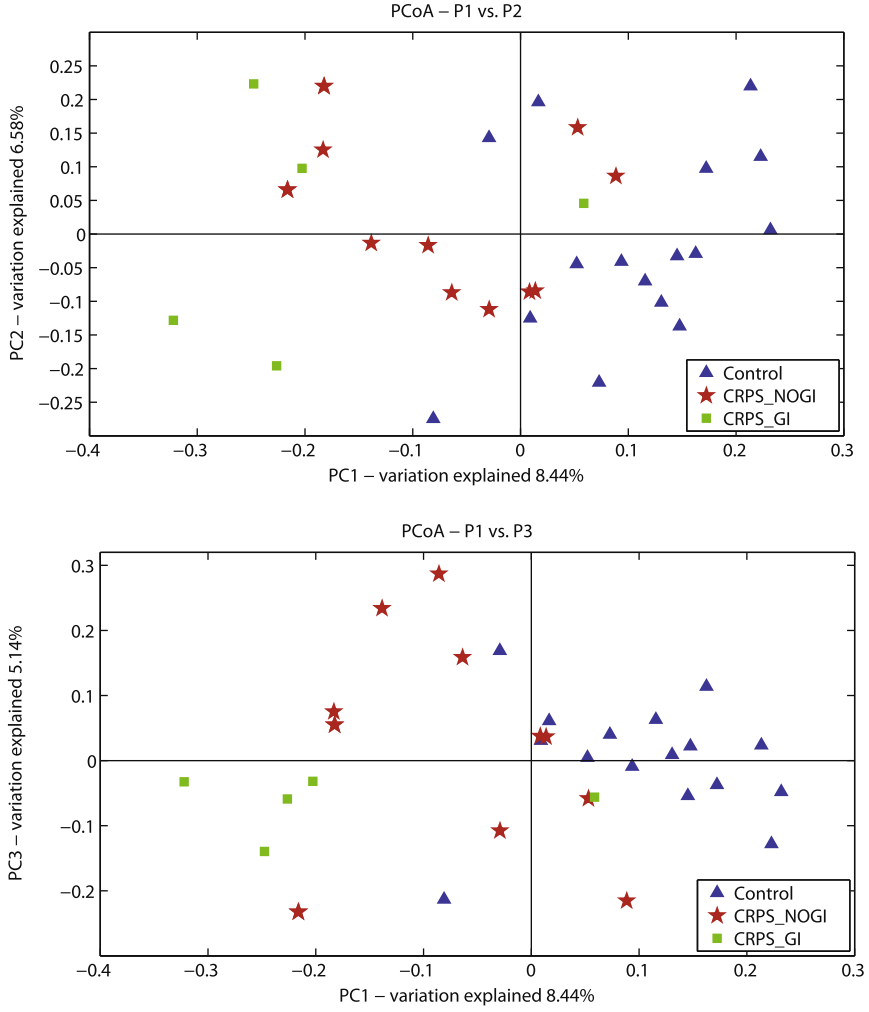


**Fig. 3.** Principal coordinate analysis (PCoA) generated from the unweighted UniFrac matrix of OTUs of CRPS subjects with gastrointestinal difficulties (CRPS_GI, $n = 5$), CRPS study participant's with no gastrointestinal difficulties (CRPS_NOGI, $n = 11$), and health controls (controls, $n = 16$).

Following the Human Microbiome Project protocol, fecal samples were used to study GI microbial communities. Bacteria DNA was extracted from fecal samples of 16 healthy controls and 16 CRPS subjects (5 with mild GI distress) by targeting the V2 region of the 16S rRNA gene. It is important to note that the differences between controls and CRPS participants are present in each type of microbial analysis: diversity, taxonomic, and spatial clustering.

CRPS subjects were found to have lower levels of diversity than their healthy counterparts. The CRPS_All group (as well as CRPS sub-groups) had significantly less species than the Control group. Rarefaction curves indicate that more species remain to be found but were congruent with diversity metrics and showed a reduction in the number of species in the CRPS_All group when compared to controls. It is unclear whether the gastrointestinal tract of CRPS study participants have always contained less bacterial diversity throughout their lives, if the reduction occurred after the development of the syndrome, or if diversity levels were a result of ingesting medications (e.g. antibiotics) consistently at some point earlier in their lives. What is known is that the decreased diversity of the GI microbial community has long been marked by GI inflammation, damage to the mucosal cover of the GI epithelium and uncontrolled production of pro-inflammatory cytokines (Manichanh et al., 2006; Frank et al., 2007; Round and Mazmanian, 2009; Walker et al., 2011). It has been suggested that rather than a particular pathogenic bacterium, the cause of GI inflammation is the result of a disruption to the phyla distribution of a healthy gut (Round and Mazmanian, 2009; Walker et al., 2011).

The presence of bacteria in such close proximity to the epithelial layer of the gastrointestinal tract is a likely contributor to GI lymphoid tissue constituting 70% of the immune cells in the human body (Van den Abbeele et al., 2011). This interaction spurs the production of luminal secretions of antimicrobial proteins from epithelial and plasma cells (Round and Mazmanian, 2009; Hooper, 2009). In gnotobiotic studies of mice and zebrafish, researchers have found that microbial absence led to modulated cytokine production, reduced epithelial mucosal integrity, defects in 'gut-associated lymphoid tissue', and an inability to mount an appropriate immune response when exposed to pathogens (Round and Mazmanian, 2009; Rawls et al., 2004; Shanahan, 2002; Forsythe et al., 2010; Hooper, 2009; Kriegela et al., 2011).

The probability of seeing a particular bacterium occur at the same rate in two study groups was assessed using RDP's LibCompare tool. The microbial libraries were significantly different ($p \ll 0.01$) in the Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Synergistetes, and Verrucomicrobia phyla between CRPS subjects and healthy controls.

When viewing the relative abundance at the phylum level, the overwhelming contributors were Firmicutes and Bacteroidetes which is in accordance with previous investigations into GI community structures (Eckburg et al., 2005; Ley et al., 2006). However, the Firmicutes and Proteobacteria contributions were significantly different between the CRPS and Control groups. It is interesting to note that CRPS subjects contained greater amounts of Proteobacteria, which is comprised entirely of gram-negative bacteria. The cell walls of gram-negative bacteria are primarily composed of lipopolysaccharides (LPS) which promotes an innate immune response in humans consisting of cytokine production and inflammation as well as a contributor of sickness response (Watkins and Maier, 2005; Medzhitov, 2007; Amaral et al., 2008; Peri and Piazza, 2012). Of particular interest is TLR4 (Toll-like receptor), a pattern recognition receptor which is a pivotal component of the innate immune system (Amaral et al., 2008; Peri and Piazza, 2012). TLR4 recognizes microorganism elements and has been associated with inflammation as a result of tissue injury (Amaral et al., 2008; Peri and Piazza, 2012).

PCoA plots were created using an unweighted UniFrac distance matrix based on demographic factors. Visual inspection and MANOVA results showed sample clustering structured by disease state, but not by any other group characteristics (PCoA plots for age, BMI, disease duration, pain levels, and medication are not shown). This applied to when the CRPS subjects were placed into one group (CRPS_ALL) as well as into two groups (CRPS_GI, CRPS_NOGI). In both cases, univariate ANOVA found the PCoA1 axis responsible for the delineation of the control and CRPS populations. While these plots showed the control samples as having less intra-variance, the same cannot be said for the CRPS subject samples – although there is a slight grouping of the CRPS_GI subjects on the PCoA P1 vs. P3 plot. There were no significant differences in the MANOVA results within the sub-groups. This clustering may be indicative of altered disease mechanisms. However, an increased sample size would be required to enhance the credence of this idea, as the existing sample sizes were too small to make overreaching conclusions.

This study is the first to report significant differences in the GI microbial community of CRPS subjects. Although the relationship between CRPS and GI bacteria has not been fully established, expanding our view of the influence microbes have on human health seems a logical step. Microbial communities have the potential to be used as a biomarkers and could help identify disease subgroups, measure disease state, elucidate disease mechanisms, and yield potential treatments. Despite the small sample size, there is sufficient evidence to warrant further research. A recent study by Kriegala et al., indicated a relationship between CRPS and certain human leukocyte antigen (HLA) haplotypes (van Rooijen et al., 2012). In future studies we would like to combine our finding of the bias in microbial populations in the GI tract of CRPS patients with this finding regrading their biased HLA usage. It would be interesting to determine to what extent the HLA bias in CRPS patients predisposes them to present specific antigens and eptiopes of the GI microbiota, and if this can be related to the changes in the microbiota and in the immune reaction to it. This is an imminently computable task as good computational models exist that can derive the HLA specific epitopes of any bacteria or virus given its sequence structure (Ginodi et al., 2008). We could thus through a set of shotgun sequencing experiments, compare both the biota population changes and how they appear to the immune system via their epitopes. We would then relate this to the HLA type of the patients and their CRPS outcome as well as their general immune function (as determined by cytokine levels and neutrophil counts). In this fashion we will combine the microbial outlook presented here with it's immune counterpart and show how these two dynamic and interconnected systems together influence the neural system and generate CRPS.

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