Anaerobe 16 (2010) 444–453

Contents lists available at ScienceDirect

Anaerobe

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

Molecular biology, genetics and biotechnology

Pyrosequencing study of fecal microflora of autistic and control children

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ARTICLE INFO

Article history:
Received 20 May 2010
Received in revised form
16 June 2010
Accepted 20 June 2010
Available online 9 July 2010

Keywords:
Pyrosequencing
Fecal microflora
Autism

ABSTRACT

There is evidence of genetic predisposition to autism, but the percent of autistic subjects with this background is unknown. It is clear that other factors, such as environmental influences, may play a role in this disease. In the present study, we have examined the fecal microbial flora of 33 subjects with various severities of autism with gastrointestinal symptoms, 7 siblings not showing autistic symptoms (sibling controls) and eight non-sibling control subjects, using the bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) procedure. The results provide us with information on the microflora of stools of young children and a compelling picture of unique fecal microflora of children with autism with gastrointestinal symptomatology. Differences based upon maximum observed and maximum predicted operational taxonomic units were statistically significant when comparing autistic and control subjects with p-values ranging from <0.001 to 0.009 using both parametric and non-parametric estimators. At the phylum level, *Bacteroidetes* and *Firmicutes* showed the most difference between groups of varying severities of autism. *Bacteroidetes* was found at high levels in the severely autistic group, while *Firmicutes* were more predominant in the control group. Smaller, but significant, differences also occurred in the *Actinobacterium* and *Proteobacterium* phyla. *Desulfovibrio* species and *Bacteroides vulgatus* are present in significantly higher numbers in stools of severely autistic children than in controls. If the unique microbial flora is found to be a causative or consequent factor in this type of autism, it may have implications with regard to a specific diagnostic test, its epidemiology, and for treatment and prevention.

1. Introduction

Autism is a complex disorder and probably embraces a number of differing entities. There are no specific diagnostic tests so the disease is defined by its characteristics—cognitive defects, including impairment of spoken and/or receptive language; social, communication and behavioral problems; repetitive behaviors; unusual sensitivity to stimuli such as noises; and restricted interests [1]. Autism research to date has mainly focused on finding a genetic association but it has been recognized that, in addition to heritable predisposition to the disease [2], environmental factors are likely important [3]. There has been a striking increase in incidence of autism worldwide; CDC's Autism and Developmental Disabilities Monitoring (ADDM) Network released data that noted that approximately 1% (1 in 110) of children in multiple areas of the U.S. in 2006 had an autistic spectrum disorder, up 57% from 2002 [4]. This increase has not been explained in a satisfactory way. We are inclined to feel that changes in diagnostic criteria and increased attention to autism in the media fail to satisfactorily explain the marked increased in incidence. It should be noted that autistic

Abbreviations: bTEFAP, bacterial tag encoded FLX amplicon pyrosequencing; OTU, operational taxonomic units, CCG, Clostridium clostridioforme group.

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doi:10.1016/j.anaerobe.2010.06.008

Table 1
Diversity and richness data for groups of subjects in the study. Data are presented at the 1% divergence level (corresponding roughly to the strain of bacteria), the 3% divergence level (corresponding roughly to the species level) and the 5% divergence level (corresponding roughly to the genus level) for rarefaction maximum predicted (RFM), ACE, and Chao1 estimates. The *p*-values (*p*-val) corresponding to a *t*-test evaluation, indicate that the controls have significantly lower numbers of operational taxonomic units than the autistic subjects.

Group means	RFM 1	RFM 3	RFM 5	ACE 1	ACE 3	ACE 5	Chao1 1	Chao1 3	Chao1 5
Mild autism mean	886	558	376	2627	1181	584	2265	1055	562
Mild autism st. dev	417	284	192	1519	680	326	1298	607	329
Severe autism mean	914	564	375	2402	1122	567	2135	1052	546
Severe autism st. dev	240	150	107	665	330	192	583	297	186
All autism mean	871	542	364	2455	1118	561	2142	1018	541
All autism st. dev	352	238	161	1250	565	274	1065	503	273
Control mean	491	296	209	1234	567	308	1092	530	300
Control st. dev	64	66	39	462	209	78	318	167	70
Sib control mean	1120	704	473	3032	1435	740	2694	1331	732
Sib control st. dev	319	237	155	1079	529	256	883	461	259
Group student's *t*-test *p*-values									
Severe vs control	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001
Control vs all aut	0.002	0.003	0.005	0.005	0.005	0.007	0.005	0.005	0.009
Control vs sib control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Sib vs severe aut	0.068	0.071	0.064	0.071	0.070	0.060	0.062	0.067	0.047

children are susceptible to various infections, particularly ear infections and that there is considerable use of antibiotics in these children. Indeed, anecdotally, parents have noted the onset of regressive disease in their children after the use of antibiotics. This could account for the changes in bowel flora to be noted in this manuscript. As with other infections involving the gastrointestinal tract, there may be transmission of infectious agents to other children who come in contact with the index case. The financial impact of autism on U.S. society is dramatic; it costs about $3.2 million to take care of an autistic person over his or her lifetime. Caring for all people with autism costs an estimated $35 billion per year according to a Harvard School of Public Health Press Release [5]. A recent consensus report from a multidisciplinary panel concluded that evidence-based recommendations for evaluation, diagnosis, and treatment of gastrointestinal disorders in subjects with autism are not yet available and that these individuals deserve the same thoroughness and standard of care for diagnostic workup and treatment as patients without autism spectrum disorders [6]. The Interagency Autism Coordinating Committee (IACC) points out the need for additional studies on the role of the environment, of epidemiology, and of specific treatments for autism [7]. Published data lend credence to the notion that an alteration in bowel microflora is associated with autistic symptoms [8–12]. The studies presented herein are concerned with detailed analysis of the fecal microflora of children with autism with gastrointestinal abnormalities.

2. Materials and methods

2.1. Subjects

33 Autistic subjects, 7 non-autistic siblings and 8 control subjects considered here were enrolled in the study at the Evergreen Center in Oregon City, OR and the Center for Autism and Related Disorders (CARD) in Tarzana, CA with signed, informed consent of their parents or guardians and approval of VA Greater Los Angeles Healthcare System Institutional Review Board IRB B. All autistic children participating in this study had an educational or developmental pediatrician evaluation and were diagnosed with autistic spectrum disorder. Subsequently, JAG (author Green) evaluated each patient for autism and validated the diagnosis based on impairment in social skills, impairment in language skills and verbal communication, sensory disturbances, repetitive stereotypical behaviors, and gastrointestinal disturbances. JAG's practice has been limited to autistic spectrum disorders since 1999 and he has evaluated and treated approximately 2000 patients in that time. All autistic and control subjects were between 2 and 13 years of age. Among the autistic group, there were 24 males and nine females. Among the control group, there were five males and three females. The gastrointestinal symptoms encountered were primarily constipation, with or without "compensatory" diarrhea, but abdominal distention and discomfort or pain were also common. The sibling control group consisted of five females, and two males. Based upon clinical

Table 2
Bacterial composition at the phylum level for control, sibling control, mildly autistic and extremely autistic subjects (3 autistic children were not considered because of unknown severity). The phylum designation is shown under the "Phylum" column. The next four columns display the percentage at which the specified phylum can be found in a specific sample with a standard deviation preceded with a "±". Non-existing standard deviations are designated with a 0.0. Control samples are designated under "Control", sibling controls are in the "S-control" column, levels for mildly autistic subjects are under "Mild-autism", while samples from the severely autistic are in the "Severe autism" column. Phyla not found in a group of samples are designated with a 0.0. The *t*-test based *p*-value is listed in the *p*-value column.

Phylum	Control ($n=8$)	S-control ($n=7$)	Mild-autism ($n=19$)	Severe autism ($n=11$)	*p*-Value severe aut vs control
Firmicutes	63.631 ± 17.593	44.012 ± 24.576	38.975 ± 15.434	38.015 ± 13.772	0.001
Actinobacteria	1.812 ± 1.679	1.037 ± 1.515	0.732 ± 1.426	0.464 ± 0.597	0.012
Bacteroidetes	30.226± 16.413	44.326 ± 17.794	51.591 ± 12.327	51.248 ± 7.043	0.001
Proteobacteria	0.535 ± 0.428	2.327 ± 3.789	2.281 ± 2.414	3.122 ± 2.579	0.011
Verrucomicrobia	5.031 ± 7.920	9.498 ± 13.214	8.092 ± 7.968	8.079 ± 11.990	0.227
Cyanobacteria	0.318 ± 0.178	0.256 ± 0.408	0.090 ± 0.117	0.069 ± 0.075	0.099
Fusobacteria	0.081 ± 0.0	0.0	0.024 ± 0.010	0.024 ± 0.0	0.194
Tenericutes	0.0	0.110 ± 0.079	0.789 ± 0.117	0.167 ± 0.209	0.098
Lentisphaerae	0.0	0.0	0.037 ± 0.0	0.0	0.0

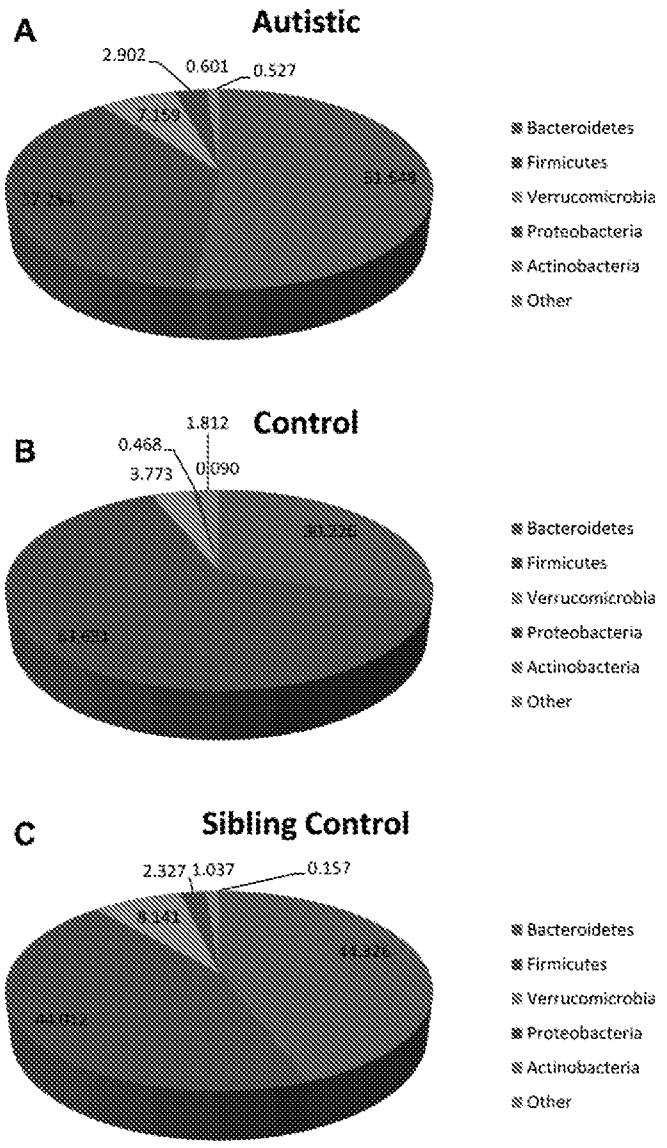

Fig. 1. Bacterial composition at the phylum level of all autistic, control and sibling control samples. Bacteroidetes and Firmicutes phyla encompass the majority of the bacteria found in fecal samples of autistic, control, and sibling control children. Just over 50% of the microflora in autistic children is comprised of Bacteroidetes while the control samples show only about 30%. Samples taken from sibling control show about 44% Bacteroidetes showing a more similar proportion of Bacteroidetes and Firmicutes to the autistic children than the controls.

evaluation we were able to distinguish the severity of autism in 30 of the 33 autistic subjects. Eleven subjects were categorized as severe, while 19 subjects were grouped into the mildly autistic category. Subjects were not eligible for the study if they had been on antibiotics or probiotics within the preceding month. It was not possible to obtain parents' consent for discontinuation of antifungal agents for one month prior to the study and it was not possible to control for the effect of diet; a number of children had special diets such as casein-free, gluten-free or specific carbohydrate diets. We plan to study the effect of diet in future studies.

2.2. Stool collection and transport

Specimens (the entire fecal specimen) were collected at the homes of the participants and were shipped to the Wadsworth Anaerobe Laboratory in Los Angeles the same day, by air express, packed with frozen blue shipping packets. The specimens always arrived in Los Angeles the next morning. In the laboratory, the specimens were thawed, homogenized in a Waring blender, and DNA was extracted. The DNA specimens were then kept frozen at −80 °C. for possible further studies.

2.3. DNA extraction

One ml aliquots of stool, previously diluted 1:3 in sterile bi-distilled water and thoroughly homogenized under anaerobic conditions in an anaerobic chamber were centrifuged at 14,000 × *g* for 3 min to pellet fecal bacterial cells. The supernatant was carefully removed and discarded. Two hundred milligrams of cell pellet was transferred to a fresh tube and subjected to DNA extraction using a commercial extraction system (QIAamp DNA stool mini kit; Qiagen) according to the instructions of the manufacturer. Previous studies in our laboratory have shown that the QIAamp product produces high-quality DNA free of PCR-inhibiting substances. DNA extraction was performed in duplicate.

2.4. Blinding of information

Information on which specimens came from autistic children and which from control children was withheld from the investigators doing the pyrosequencing until their data set was completed. Subsequently, this information was provided to them by the group in Oregon so that proper group analyses of the data could be completed.

2.5. bTEFAP

We utilized bTEFAP with titanium chemistry, as described previously by the Dowd laboratory [13–17], to evaluate the bacterial populations in the feces of separate autistic children in comparison to individual control samples. In preparation for FLX sequencing (Roche, Nutley, New Jersey), the DNA fragments' size and concentration were accurately measured. A small sample of double-stranded DNA molecules/μl was combined with DNA capture beads, and then amplified by emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNAs were denatured with NaOH, and sequencing primers were annealed. A 454 sequencing run was performed using the Genome Sequencer FLX Titanium System (Roche, Nutley, New Jersey). All FLX Titanium procedures were performed using Genome Sequencer FLX System manufacturer's instructions (Roche, Nutley, New Jersey).

2.6. bTEFAP sequence processing pipeline

Custom software written in C# within a Microsoft® .NET (Microsoft Corp, Seattle, WA) development environment was used for all post-sequencing processing. Quality trimmed sequences obtained from the FLX Titanium sequencing run were derived directly from FLX Titanium sequencing run output files. Tags were extracted from the multi-FASTA file into individual sample-specific files based upon the tag sequence. Sequences which were less than 350 base pairs after quality trimming were not considered. Sequences were analyzed by a script optimized for high throughput data. Definite chimeras removed using B2C2 (software are available at http://researchandtesting.com/B2C2). The resulting FASTA for each sample, with chimeras removed, were then evaluated using BLASTn [18, 19] against a custom database derived from NCBI, curated based upon quality criteria similar to that utilized for high-quality sequences of the RDP-II database [20]. C# scripts were used to extract necessary taxonomic information from NCBI for the accession numbers derived from the database queries.

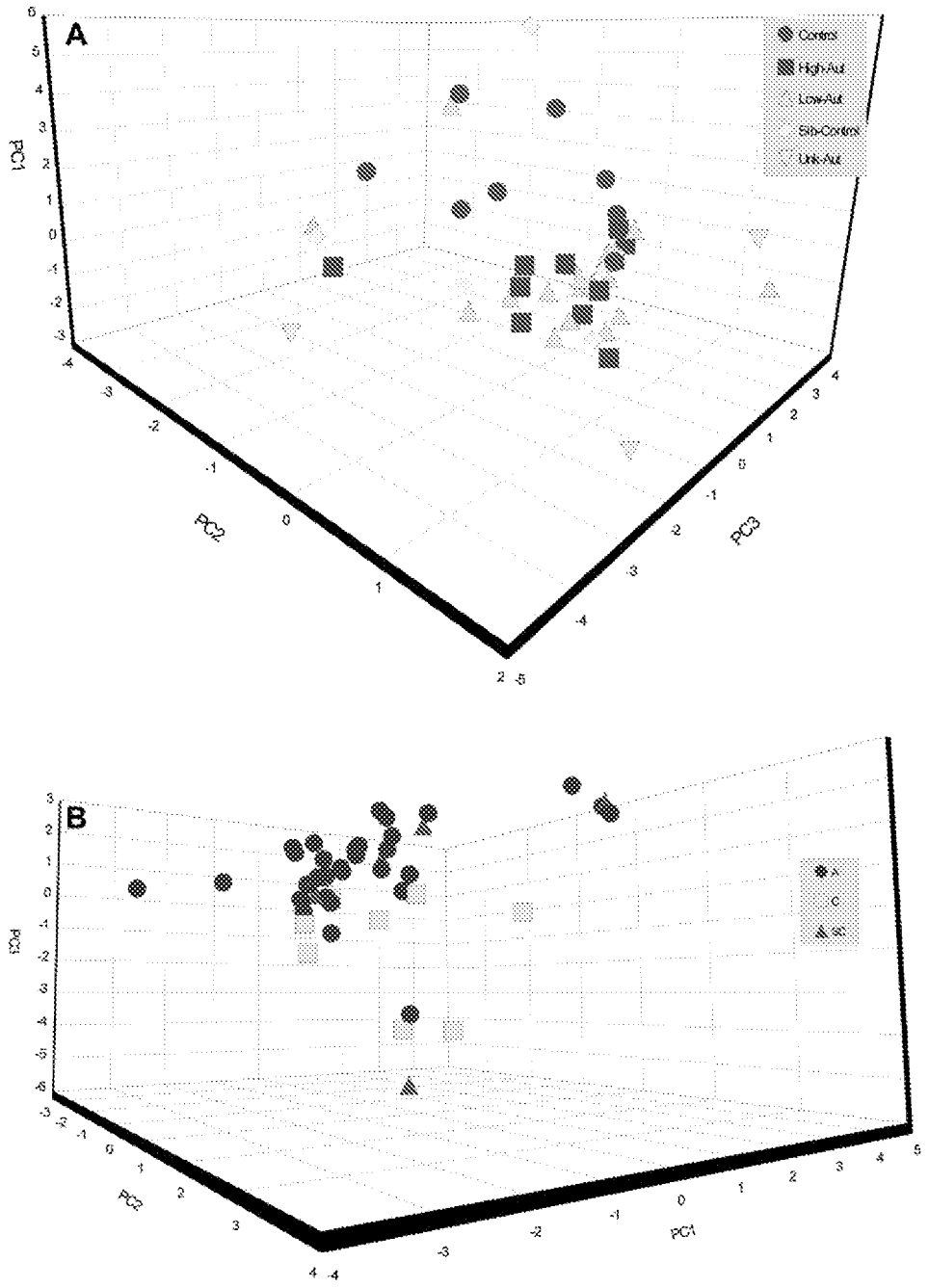

Fig. 2. Principal Component Analysis of the phylum information for autistic, sibling control and control groups. The three principal components cover 60.83% of the variation in the data of A and 55.31% in B. A. shows results for all the groups separated by severity while B groups all autistic children together. Based on phylum information, the autistic samples appear to cluster together, regardless of the severity of autism. The sibling controls are mixed into the autistic cluster indicating a similarity in the microflora. The true control samples, however, are separated from the other samples and are less close together.

Microbial diversity analysis [21–24] was performed by clustering sequence tags into groups of defined sequence variation ranging from unique sequences (no variation) to 10% divergence evaluated, as previously described, from raw reads of comparable Phred20 quality (>350bp). [25]. Clusters acting as OTUs were used to generate rarefaction curves and as input for calculations with the abundance-based coverage estimator ACE and the Chao1 [26] estimator of species diversity. Table 1 shows the microbial diversity estimate averages and *t*-test results obtained with (parametric and non-parametric) modeling of rarefaction, ACE and Chao1. Final datasets classified at the species and other relevant taxonomy levels were compiled into separate worksheets. To assess not only the overall bacterial richness of the samples, but the actual populations, we conducted a "composition analysis". This process produced results containing information for each sample at each taxonomic level (kingdom, phylum, class…).

3. Statistics

3.1. Principal component analysis

To assess the separability of the samples, Principal Component Analysis was implemented. Principal Component Analysis (PCA) [27] is widely used for dimensionality reduction to help with

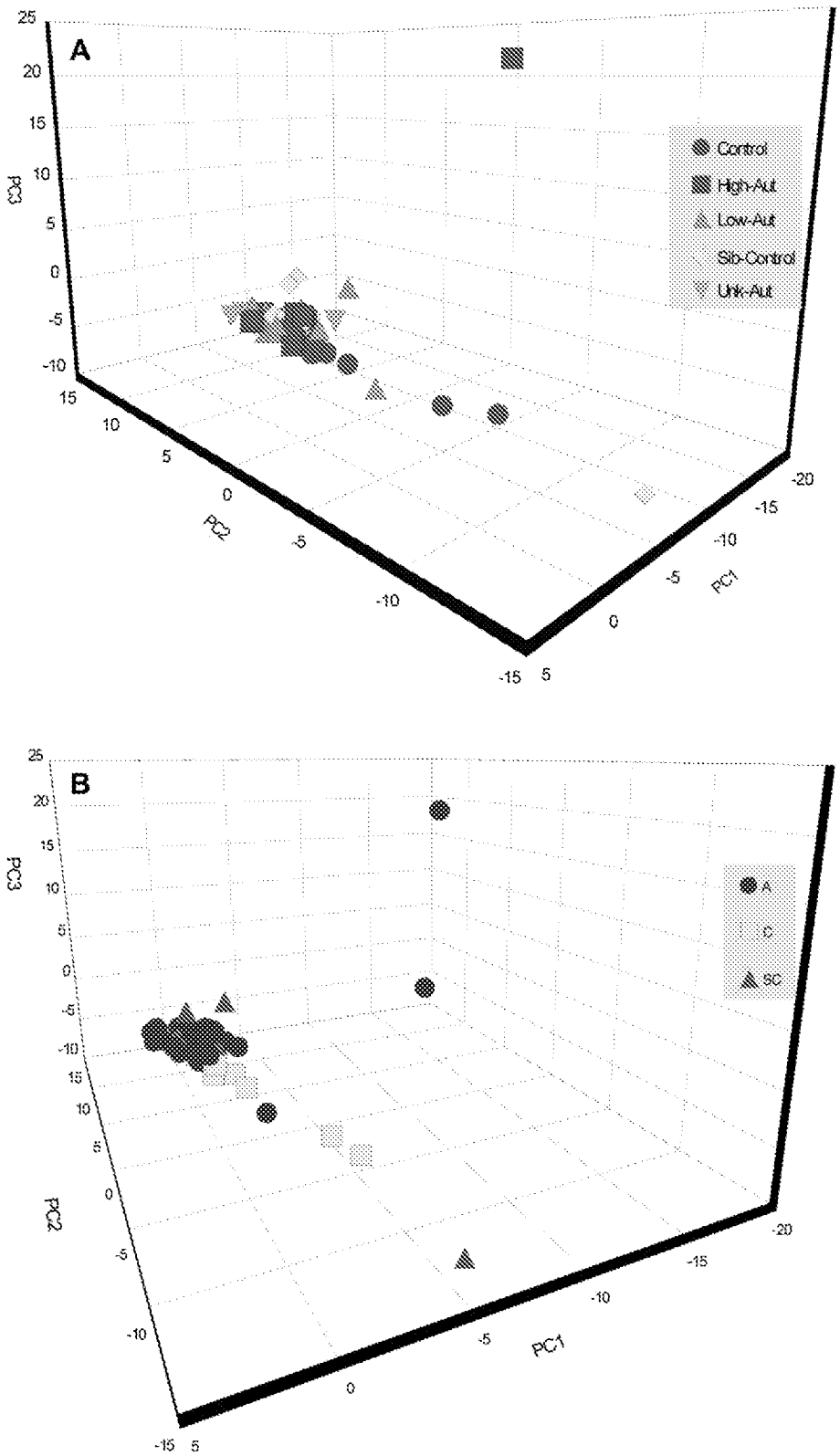

Fig. 3. Principal Component Analysis of the genus information for autistic and control children. A. shows results for all the groups separated by severity while B groups all autistic children together. The three principal components cover 18.46% of the variation. The autistic samples appear to cluster together, regardless of the severity of autism. The sibling controls are mixed into the autistic cluster indicating a similarity in the microflora. The true control samples, however, are separated from the other samples and are less close together.

Table 3
Bifidobacterium spp. quantities and significance levels within severely autistic and control samples (non-sibling). *Bifidobacterium* species are listed in the "Species" column, along with the average percentage at which they were found in the autistic and control samples. These values are listed in the "Avg A" and "Avg C" columns for autistic and control samples, respectively, followed by their respective standard deviations listed in the "St. dev" columns. A *t*-test based *p*-value is listed under the "*p*-value" column.

Species	Avg A ($n = 11$)	St. dev A	Avg C ($n = 8$)	St. dev C	*p*-Value
B. adolescentis	0.125	0.261	0.154	0.384	0.424
B. angulatum	0.000	0.000	0.046	0.089	0.050
B. animalis	0.005	0.016	0.000	0.000	0.205
B. bifidum	0.012	0.020	0.017	0.048	0.372
B. dentium	0.001	0.005	0.000	0.000	0.205
B. longum	0.084	0.150	0.636	0.957	0.037
B. pseudocatenulatum	0.025	0.063	0.161	0.288	0.072
B. pseudolongum	0.000	0.000	0.012	0.034	0.126
B. saeculare	0.006	0.020	0.000	0.000	0.205
Bifidobacterium genus	0.258		0.409		0.050

visualization of high dimensional data. PCA is defined as the orthogonal projection of the data onto two or three dimensional space such that the variance of the projected data is maximized. Custom Python scripts tailored for next generation data (distance matrices and taxonomic abundance) were implemented to assess bacterial composition of samples and determine the 3 Principal Components. This data is visualized by plotting the samples on axes defined by the principal components. Samples more similar to each other should appear closer together according to the respective axis reflecting the variation among all samples. This technique is useful in displaying clusters existing within data. The variables (features) are the relative bacterial composition in a sample at a particular taxonomic level.

3.2. Clustering

To analyze the relationships and clustering between autistic and control samples, double dendrograms were formed based on the bacteria composition information. The analysis was performed using the NCSS Statistical Software as described previously [13,15,25,28–30].

3.3. Other statistics

As appropriate, student's *t*-tests were used for comparing means within various groups of data.

4. Results and discussion

The results indicate there is a significantly higher diversity of bacteria found in the feces of autistic subjects compared to controls (Table 1). Even when relatively large genetic distances (5% divergence) are considered, these estimates predict that even at the genus level there is significantly less diversity (at a 5% significance level) and richness of microbial communities in control subjects than in the autistic group. These diversity results were similar when using three separate diversity and richness methods (including Chao1, ACE, and rarefaction). From this data we see that the parametric method of rarefaction [25] predicts that the average number of operational taxonomic units present in the feces of all autistic samples at 3% sequence divergence (the species level) was 542 compared to 296 in the control samples. Using the ace non-parametric measure of richness, we see that there are a predicted 1118 species in the autistic samples and 567 in the control samples and based upon Chao1 there were an average of 1018 and 530, respectively. This dramatic and significantly increased diversity and

Table 4
Top 20 occurring genera out of 198 of severely autistic and control (non-sibling) subjects. The number of samples the bacterial species were seen in is listed in the # A or #C columns. The average percentage (% Total Flora) designates the average percentage of the specific taxa found in the total microflora in the group of samples containing the genus (autistic or control).

A, top 20 genera	# A ($n = 11$)	% Total Flora A	C, top 20 genera	# C ($n = 8$)	% Total flora C
Bacteroides	11	35.544	Bacteroides	8	24.481
Clostridium	11	10.343	Clostridium	8	17.748
Faecalibacterium	11	10.173	Faecalibacterium	8	11.271
Eubacterium	11	5.521	Ruminococcus	8	7.581
Ruminococcus	11	3.329	Eubacterium	8	9.749
Roseburia	11	2.033	Alistipes	8	2.621
Dorea	11	0.297	Roseburia	7	0.742
Hespellia	11	0.176	Anaerofilum	5	0.104
Turicibacter	11	0.152	Streptococcus	8	0.600
Akkermansia	10	7.344	Turicibacter	6	3.773
Parabacteroides	10	5.222	Parabacteroides	7	1.980
Alistipes	10	4.296	Dorea	8	3.504
Sporobacter	9	1.173	Veillonella	6	0.740
Bifidobacterium	9	0.258	Akkermansia	5	1.026
Anaerostipes	9	0.223	Sporobacter	1	0.054
Ethanoligenens	9	0.113	Ethanoligenens	6	0.477
Anaerotruncus	9	0.092	Papillibacter	5	0.140
Holdemania	9	0.084	Holdemania	6	0.107
Phascolarctobacterium	8	1.382	Weissella	4	1.918
Desulfovibrio	8	0.276	Dialister	3	0.032

richness may be an important aspect of the autistic gastrointestinal microbiome. The increased microflora of autistic children may contain harmful genera or species contributing to the severity of autistic symptoms. *Bacteroidetes* and *Firmicutes* are shown to be important phyla in this analysis. It should be noted that the vast majority of species in the *Bacteroidetes* produce propionic acid and other short-chain fatty acids as endproducts of their metabolism and MacFabe and colleagues have shown clearly that when propionic acid or other short-chain fatty acids are injected into cerebral ventricles of rats, the rats show biologic, chemical, and pathologic changes that are characteristic of autism [31]. It should also be noted that these organisms, particularly the *Bacteroides fragilis* group, produce lipopolysaccharide (LPS), an important virulence factor; *Desulfovibrio* also produces LPS as well as hydrogen sulfide which can be a potent poison under certain circumstances. *Bacteroides vulgatus* has been implicated in ulcerative colitis, as has *Desulfovibrio*. Decreasing harmful populations with antibiotics like vancomycin has been shown to be an important step in improving late onset autism symptoms [8].

The OTU data also indicates no statistically significant difference between the sibling control subjects and the severely autistic subjects. However, when comparing the true controls against sibling controls, the estimated richness does prove to be statistically different. This and other tests discussed later indicate the sibling controls to be more similar to autistic children than to the true control subjects. This raises the question of possible transmission of fecal bacteria from cases of autism to siblings (multiple cases of autism are not rare in families), playmates, etc. It is easy to visualize fecal contamination of surfaces and fomites from diaper-clad infants with autism who may be even less hygienic than healthy children of that age. Another child encountering these organisms (e.g., contaminating their hands and then putting their fingers in their mouth) could become colonized and, if predisposed by a faulty immune system and an abnormal bowel flora related to antimicrobials, could develop autism. This type of scenario may be an explanation for the remarkable increase in incidence of this disease in the antibiotic era. We had previously speculated on this possibility in relation to clostridia, especially the *Clostridium clostridioforme* group [12]. The study currently

Table 5
Significant genera among severely autistic vs non-sibling control samples. The number of samples of the specific bacterial genus found is listed in the "# of Autistic" or "# of Control", depending on which group the bacteria was found in exclusively. The average percent of bacteria found is in the column "Avg % A" or "Avg % C" for autistic or control samples, respectively; "0.000" indicates undetected. *p*-Values are provided for comparisons of A vs C for each of the genera specified. A total of 198 genera were considered. Genera listed in bold are in the top 20 most predominant genera in extremely autistic or control groups (Table 4).

Genus	# of Autistic ($n = 11$)	# of Control ($n = 8$)	avg % A	avg % C	*p*-Val A vs C
Weissella	0	6	0.000	0.095	<0.001
Turicibacter	11	8	0.152	0.600	<0.001
Clostridium	11	8	10.343	17.748	0.001
Anaerofilum	8	8	0.240	1.228	0.005
Alkaliflexus	8	0	0.122	0.000	0.006
Pseudoramibacter	5	5	0.027	0.132	0.011
Desulfovibrio	8	3	0.276	0.032	0.011
Acetanaerobacterium	8	1	0.083	0.005	0.015
Ruminococcus	11	8	3.329	9.749	0.018
Streptococcus	8	8	0.135	0.861	0.019
Anaerovorax	4	5	0.017	0.159	0.028
Dialister	4	5	0.090	4.691	0.035
Lactococcus	0	3	0.000	0.028	0.035
Parabacteroides	10	7	5.222	1.980	0.036
Leuconostoc	4	3	0.010	0.052	0.040
Ethanoligenens	9	6	0.113	0.477	0.041
Bacteroides	11	8	35.544	24.481	0.044
Helcococcus	0	2	0.000	0.011	0.045
Alkaliphilus	0	2	0.000	0.010	0.046

being reported involves a larger number of stools from both autistic subjects and controls and the pyrosequencing technique permits detection of many times the number of bacteria that can be demonstrated by cultural techniques and PCR procedures and therefore gives us a better picture of the flora of these subjects. Nevertheless, there are other spore-forming bacteria and other non-spore-formers that resist dessication and death, particularly if encased in fecal material.

Summary information at the phylum level for all four groups of samples (severely autistic, mildly autistic, control and sibling control) are shown in Table 2. There are also significant differences between severely autistic subjects and controls with regard to the *Actinobacterium* and *Proteobacterium* phyla. A trend can be seen in *Firmicutes* where the level of this phylum is much higher in the control than autistic samples. Taxonomically, it is not surprising at the phylum level that *Bacteroidetes* (Table 2) were significantly higher in counts in autistic subjects (p 0.001) while *Firmicutes* tended to be higher in the control subjects (p 0.001). These data point to an altered microflora in the gut of autistic subjects. Fig. 1A–C shows the composition of autistic and control and sibling control samples, respectively, and again emphasize the difference between the autistic and control groups. The sibling control figure (Fig. 1c), proportionally looks to be between the autistic and control groups, as might be expected. However, similar to the autistic group, *Firmicutes* comprises less than 50% of the bacteria, unlike in the control group where *Firmicutes* represents an average of 63.6%.

Using the phylum composition data to analyze the microbiome further, Principal Component Analysis was performed. The analysis incorporates 9 variables (the nine phyla represented in the samples). The data suggested the autistic and control individuals were separable based on the taxonomic percentages associated with each of the groups (Fig. 2A). Fig. 2A displays the mapping of all four classes at the phylum level and covers 60.935 per cent of the variation. The image accentuates the difference of the control samples from the rest of the groups. While the control points are more scattered across the grid, the mild and severe autistic points, along with the siblings of autistic children tend to cluster together. This can be more clearly seen in Fig. 2B where all autistic samples are grouped under the same color. This supports the supposition that there is a difference in the fecal microflora between autistic and non-autistic children. Even children close to autistic individuals seem to possibly be influenced by the bacteria, and overall do not appear to be statistically different from children suffering from autistic symptoms.

A similar graphical pattern of separation for the four groups propagates through all taxonomic levels. In Fig. 3A and B, the PCA results for the genus level (198 variables/genera used for analysis) can be seen. Although the dividing line was less obvious and the three principal components only covered 18.46% of the variation, the control samples remained distinguishable from the rest of the samples.

Another observation associated with abundance data at the genus level indicated that there are reduced populations of the *Bifidobacterium* genus in the severely autistic samples, compared with the controls. Evaluation of the *Bifidobacterium* genus-related data shows that there was a significantly higher occurrence of

Table 6
Genera and species present in greater than 1% of the total flora in one or more groups of children.

	Autistic (no)		Control (no)	
	Mild (22)	Severe (11)	Normal (8)	Sibling (7)
	% Of the total flora			
Firmicutes:				
Clostridium aldenense	1.9	1.7	1.7	0.6
Clostridium hathewayi	2.3	1.6	1.2	1.6
Clostridium leptum	0.2	0.4	2.7	0.1
Clostridium methylpentosum	0.3	0.1	1.6	0.2
Clostridium orbiscindens	1.8	1.7	1.3	0.6
Dialister invisus	0.6	0.1	4.7	1.1
Eubacterium eligens	1.9	3.0	0.6	1.7
Eubacterium ruminantium	0.2	0.1	1.7	1.4
Phascolarctobacterium faecium	1.5	1.4	1.9	2.2
Roseburia intestinalis	3.6	2.0	2.5	2.4
Sporobacter termitidis	1.0	1.2	0.7	1.5
Bacteroidetes:				
Alistipes onderdonkii	1.2	1.19	1.4	0.5
Bacteroides caccae	1.7	0.5	4.9	0.9
Bacteroides stercoris	2.3	0.7	0.3	2.8
Bacteroides vulgatus	13	12	3.6	2.5
Parabacteroides distasonis	2.5	2.5	1.6	1.1
Prevotella oulorum	0.2	2.0	0	8.6

Table 7
Genera and species of possible importance in contributing to autism.

	% Of total flora		
	Severe autism (11)	Control (8)	*p*-Value
Desulfovibrio genus	0.28	0.03	0.010
Desulfovibrio piger	0.11	0.006	0.032
Desulfovibrio desulfuricans	0.28	0	0.035
Desulfovibrio intestinalis	0.10	0.03	0.045
Bacteroides vulgatus	12.13	3.63	0.045

Table 8
Genera and species of possible importance as protective flora.

	% Of total flora		
	Severe autism (11)	Control (8)	*p*-Value
Collinsella genus	0.02	0.62	0.050
Bifidobacterium genus	0.26	0.41	0.050
Bifidobacterium longum	0.08	0.64	0.037
Bifidobacterium angulatum	0	0.05	0.050
Dialister invisus	0.08	4.67	0.035
Clostridium leptum	0.35	2.70	0.010

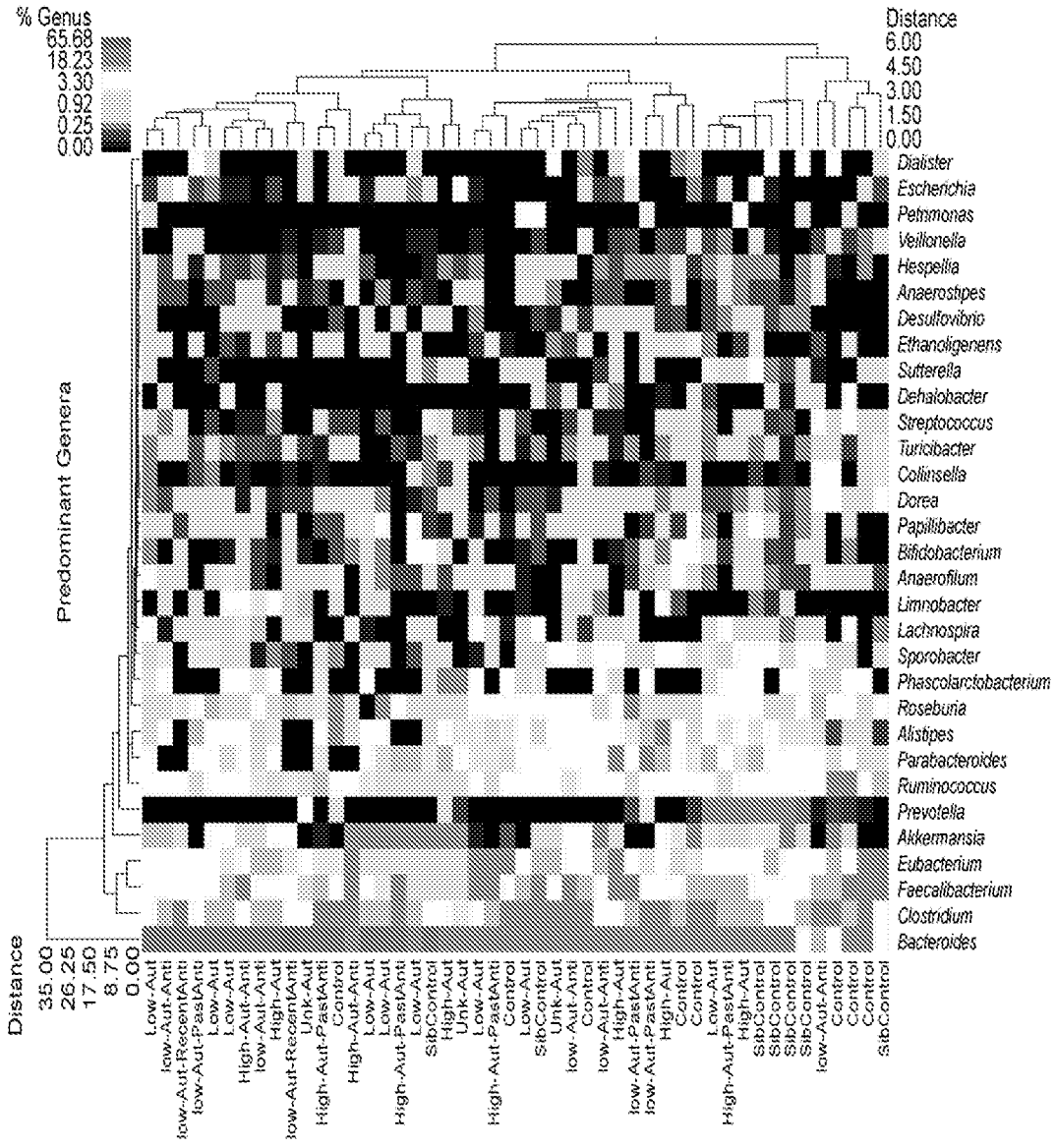

Fig. 4. Double Dendrogram representation of clustering of all samples based on genus abundance information. The severity of the autism and antibiotic history are indicated for samples when such information is available. The sibling control and true control samples are also included. The clustering shows groupings of the mildly autistic children's samples toward the left portion of the figure. Many of the control and sibling control samples cluster toward the right of the figure. This pattern indicates a differentiation in the bacterial microflora between autistic and control samples.

species among the control subjects than in the autistic subjects ($p < 0.05$) and though not individually significant, the species were found at higher frequencies in the control subjects (Table 3). *Bifidobacterium* along with *Lactobacillus* spp. are notable as probiotics, though little is known about strain specific differences, which may be host or individually specific [32]. Probiotic therapy to alleviate symptoms of gastrointestinal disorders has produced only slight [33,34] or no improvement of such disease states [32,35,36]. The use of probiotics in autism-related disorders has also been discussed in the literature [37–39] though little clinical evidence is apparent for the efficacy of such treatment. Different species of *Bifidobacterium* produce different exopolysaccharides that act as fermentable substrates for different human intestinal bacteria [40].

Other genera of interest are listed in Table 4. In this table, the top 20 genera, of a total of 198 encountered, indicated a similar overall composition between the severely autistic and control groups. However, *Hespellia*, *Anaerostipes*, and *Desulfovibrio* spp. were seen in the top 20 genera only in the autistic subjects and *Streptococcus*, *Veillonella*, *Weissella*, and *Papillibacter* spp. were only found among the top 20 in the control subjects. The 19 genera with significant differences from this data include *Turicibacter*, *Clostridium*, *Weissella*, *Parabacteroides* and *Ruminoccocus* spp. and others (Table 5). The mean differences for the other 179 genera were not significant. Table 6 shows the various genera and species detected among the *Firmicutes* and *Bacteroidetes* phyla. Tables 7 and 8 show genera and species of possible importance in contributing to the clinical picture of autism or as protective flora, respectively. *Desulfovibrio* was particularly interesting since all three species of this genus that were encountered showed significantly greater percentages of the total flora in the stools of severely autistic children than in controls although the numbers are small. Furthermore, this sulfate-reducing genus has been recovered from serious infections such as bacteremia. This genus produces hydrogen sulfide, an important virulence factor that is known to corrode various metals. It might have the opportunity to attack certain metals such as iron, mercury, molybdenum, etc. in the bowel.

Using the overall predominant genera, clustering analysis was performed to assess the importance of the bacterial gut flora of autistic children and the control and sibling control subjects. Fig. 4 shows the results of a clustering of all samples. Starting with the 198 genera, the number of genera was reduced until there was a change in the clustering. Thus only 27 of the 198 genera are needed to display the clustering patterns. The left and right sides of the double dendrogram show some indication of grouping. In the left portion of the image, the mildly autistic samples group together on the far left and other severity levels of autism further to the right. The far right portion of the dendrogram is primarily composed of the control and sibling control individuals. Based on this information, there appears to be some indication of the gut microflora differing between the autistic and control groups. The *Bacteroides* genus, in particular, is an obvious indication of the change that occurs from autistic to sibling and control children. The red color, indicating a high abundance of the genus regresses to more orange and yellow tones indicating a decrease in the amount of the bacteria.

5. Conclusions

An issue that needs to be decided is does the autism lead to the altered flora and/or does the altered flora play a role in the disease or its syndromes. In other situations with major changes in bowel microflora associated with disease (e.g., *Clostridium difficile*-asssociated pseudomembranous colitis [41] and trinitrobenzenesulphonic acid-induced colitis in mice) [42], fecal enemas or implantation of individual members of the fecal flora leads to improvement in the disease process [42,43]. Improvement in autistic subjects treated with oral vancomycin (virtually not absorbed and therefore active only in the bowel) in a small pilot study [8] and anecdotally also suggests that the altered microflora might play a role in autism syndromes. Clearly, there is a need for a double-blind, placebo-controlled trial of oral vancomycin or a similar drug. Other studies that might shed further light on the subject include chemostat (fermentor) studies, use of animal models, and flora augmentation and transplant studies. It is possible that lower levels of beneficial bacteria combined with heightened levels of harmful bacteria contribute to the ability of potentially pathogenic species to exert a stronger influence on autistic subjects.

The relative importance of immune dysfunction (genetic or environmental [including bacteria themselves], antimicrobial agents and/or diet leading to dysbiosis of the intestinal microflora, various virulence factors of bacteria) that are involved, and methods of transmission of the bacteria remain to be determined; all should be studied. In any case, it seems clear now that autism research should include study of gastrointestinal symptomatology and pathology, the immune system, the impact of various antimicrobials on the bowel flora, and intestinal bacteria and their virulence factors. To what extent the findings in this manuscript might apply to other types of autism should be determined.

It should also be noted that the findings in this study may have application to other diseases of uncertain etiology and autoimmune diseases such as rheumatoid arthritis, multiple sclerosis. Parkinson's disease, Alzheimer's disease and others.

6. Potential problems or pitfalls

A factor of major potential, not evaluated in the current study, may be the immune status of the host; there may be predisposition to suffer ill effects from an abnormal intestinal microbial flora, related to hereditary and/or environmental factors; we presume these to be present. Other factors not evaluated in this study, but potentially important are mucosa-associated microflora and the possibility of biofilm formation (we studied only luminal flora). It should also be noted that pyrosequencing, while a powerful method, is limited by normal primer biases as well as the incomplete nature of 16S rDNA databases, and the fact that other genes are not usually studied. As more bacteria are sequenced and added to the database and studies are conducted with multiple primers the effects of these limitations will decrease.

Acknowledgments

SF would like to express appreciation to Mrs. Ellen R. Bolte and Richard Sandler, M.D. who first got him interested in studying autism. The authors express their appreciation to Jeffrey Gornbein, Dr.P.H., UCLA SBCC/Dep't of Biomathematics, David Geffen School of Medicine, Los Angeles, CA for his review of the manuscript and statistical assistance.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.anaerobe.2010.06.008.

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