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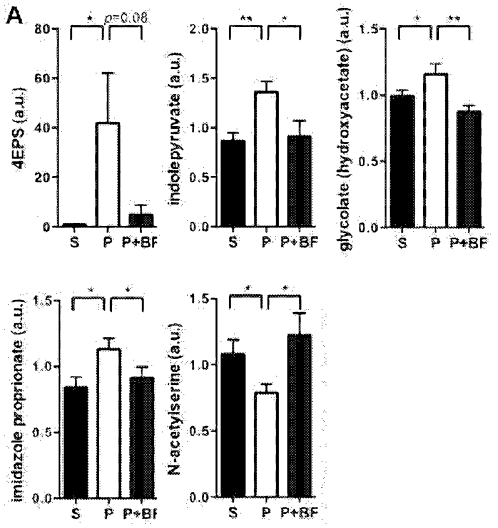
Figure 13



(57) Abstract: Disclosed herein are compositions, systems, and methods for diagnosing and treatment of subjects suffering from anxiety, autism spectrum disorder (ASD), or a pathological condition with one or more of the symptoms of ASD.

WO 2014/036182 A3

DIAGNOSIS AND TREATMENT OF AUTISM SPECTRUM DISORDER

RELATED APPLICATIONS

[0001] The present application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 61/694,679, filed on August 29, 2012, which is herein expressly incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED R&D

[0002] This invention was made with government support under grant no. W81XWH-11-0515 awarded by the Army, Graduate Training grant No. 5 T32 GM07737 awarded by National Institutes of Health, Graduate Research Fellowship No. DGE-0703267 awarded by National Science Foundation, and grant No. MH100556 awarded by National Institutes of Mental Health. The government has certain rights in the invention.

REFERENCE TO SEQUENCE LISTING

[0003] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled SEQLISTING.TXT, created August 28, 2013, which is 4 Kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND

Field of the Invention

[0004] The present application relates generally to the field of diagnosing and treatment of autism spectrum disorders (ASD).

Description of the Related Art

[0005] Autism spectrum disorder (ASD) is a serious neurodevelopmental disorder characterized by stereotypic behaviors and deficits in language and social interaction. The reported incidence of autism has rapidly increased to 1 in 88 births in the United States as of 2008 (CDC, 2012), representing a significant medical and social burden in the coming

2013308826 19 Mar 2019

decades. Reproducible molecular diagnostics for ASD have not been developed and therapies for treating the core symptoms of ASD are limited, and reproducible molecular diagnostics have not been developed. Much research into autism spectrum disorder (ASD) has focused on genetic, behavioral and neurological aspects of disease, but primary roles for environmental risk factors (Hallmayer et al., 2011), immune dysregulation and additional peripheral disruptions in the pathogenesis of ASD have recently gained significant attention. The striking heterogeneity among individuals that share the same diagnosis is consistent with the prevailing notion that there are a variety of etiologies for ASD. Moreover, the spectrum of ASD symptoms and challenges in identifying specific causes, treatments and molecular biomarkers underscore the need to better define the clinical subtypes of ASD and provide tailored treatment to subclasses of ASD individuals.

SUMMARY

[0006A] Some embodiments disclosed herein are related to a method for improving behavioral performance in a subject, where the method includes: determining the blood level of an autism spectrum disorder (ASD)-related metabolite in a subject in need of treatment; and adjusting the blood level of the ASD-related metabolite in the subject until an improvement in the behavioral performance in the subject is observed.

[0006B] In another embodiment of the present invention there is provided a method for improving behavioral performance in a subject, comprising: determining the level of one or more autism spectrum disorder (ASD)-related metabolites in the blood or urine of a subject In need of treatment; and adjusting the blood and/or urine level of at least one of the one or more ASD-related metabolites in the subject until an improvement in the behavioral performance in the subject is observed; wherein the subject in need of treatment suffers from anxiety, ASD, or a pathological condition with one or more of the symptoms of ASD.

[0006C] In a further embodiment of the present invention there is provided a method for improving behavioral performance in a subject suffering from anxiety, Fragile X, Rett syndrome, tuberous sclerosis, obsessive compulsive disorder, attention deficit disorder, schizophrenia, autistic disorder (classic autism), Asperger's disorder (Asperger syndrome), pervasive developmental disorder not otherwise

specified (PDD-NOS), or childhood disintegrative disorder (CDD), the method comprising:

determining the level of 4-ethylphenylsulfate in the blood of a subject in need of treatment, wherein said level of 4-ethylphenylsulfate in the subject is altered relative to a reference level of 4-ethylphenylsulfate observed in a subject not suffering from or showing symptoms of anxiety, Fragile X, Rett syndrome, tuberous sclerosis, obsessive compulsive disorder, attention deficit disorder, schizophrenia, autistic disorder (classic autism), Asperger's disorder (Asperger syndrome), PDD-NOS, or CDD; and

administering a composition comprising *Bacteroides* bacteria to the subject until an improvement in the behavioral performance in the subject is observed.

[0007] In some embodiments, the subject suffers from anxiety, autism spectrum disorder (ASD), or a pathological condition with one or more of the symptoms of ASD. In some embodiments, the subject suffers from ASD.

[0008] In some embodiments, adjusting the blood level of the ASD-related metabolite comprises adjusting the composition of gut microbiota in the subject. In some embodiments, adjusting the composition of gut microbiota of the subject comprises fecal transplantation. In some embodiments, adjusting the composition of gut microbiota of the subject comprises administering the subject a composition comprising *Bacteroides* bacteria. In some embodiments, the *Bacteroides* bacteria is *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, or a mixture thereof.

[0009] In some embodiments, the composition is a probiotic composition, a neutraceutical, a pharmaceutical composition, or a mixture thereof.

[Text continued on page 3]

[0010] In some embodiments, adjusting the composition of gut microbiota of the subject comprises reducing the level of *Clostridia* bacteria in the subject. In some embodiments, the *Clostridia* bacteria is *Lachnospiraceae*. In some embodiments, adjusting the composition of gut microbiota of the subject comprises increasing the level of *Ruminococcaceae*, *Erysipelotrichaceae*, and/or *Alcaligenaceae* bacteria in the subject.

[0011] In some embodiments, the ASD-related metabolite is one of the metabolites listed in Table 1. In some embodiments, the ASD-related metabolite is a metabolite involved in tryptophan metabolism, a metabolite involved in fatty acid metabolism, a metabolite involved in purine metabolism, glycolate, imidazole propionate, or N-acetylserine. In some embodiments, the metabolite involved in tryptophan metabolism is 4-ethylphenylsulfate, indolepyruvate, indolyl-3-acryloylglycine, or serotonin. In some embodiments, the ASD-related metabolite is 4-ethylphenylsulfate, indolepyruvate, glycolate, or imidazole proprionate.

[0012] In some embodiments, adjusting the blood level of the ASD-related metabolite in the subject comprises administering to the subject an antibody against the ASD-related metabolite, an antibody against an intermediate for the in vivo synthesis of the ASD-related metabolite, or an antibody against a substrate for the in vivo synthesis of the ASD-related metabolite.

[0013] In some embodiments, the ASD-related metabolite is 4-ethylphenylsulfate or indolepyruvate.

[0014] In some embodiments, adjusting the blood level of the ASD-related metabolite in the subject comprises inhibiting an enzyme involved in the in vivo synthesis of the ASD-related metabolite.

[0015] In some embodiments, adjusting the blood level of the ASD-related metabolite ameliorates gastrointestinal (GI) distress of the subject. In some embodiments, the GI distress comprises abdominal cramps, chronic diarrhea, constipation, intestinal permeability, or a combination thereof. In some embodiments, adjusting the blood level of the ASD-related metabolite reduces intestinal permeability of the subject.

[0016] In some embodiments, the method includes determining the reference level of the metabolite in non-autistic subjects. In some embodiments, the method includes

determining the behavioral performance of the subject prior to and after adjusting the blood level of the ASD-related metabolite in the subject.

[0017] In some embodiments, determining the behavioral performance of the subject comprises using Autism Behavior Checklist (ABC), Autism diagnostic Interview-Revised (ADI-R), childhood autism Rating Scale (CARS), and/or Pre-Linguistic Autism Diagnostic Observation Schedule (PL-ADOS).

[0018] Also disclosed herein in some embodiments is a method for improving behavioral performance in a subject, where the method incudes: determining the urine level of an autism spectrum disorder (ASD)-related metabolite in a subject in need of treatment; and adjusting the urine level of the ASD-related metabolite in the subject until an improvement in behavioral performance in the subject is observed. In some embodiments, the ASD-related metabolite is 4-methylphenyl, 4-methylphenylsulfate or indolyl-3-acryloylglycine.

[0019] In some embodiments, adjusting the urine level of the ASD-related metabolite comprises adjusting the composition of gut microbiota in the subject. In some embodiments, adjusting the composition of gut microbiota of the subject comprises administering the subject a composition comprising *Bacteroides* bacteria.

[0020] Some embodiments provided here are related to a method for assessing the susceptibility of a subject suffering from autism spectrum disorder (ASD) to probiotic treatment, where the method includes: determining the blood level of a *B. fragilis*-responsive metabolite in the subject; and comparing the blood level of the *B. fragilis*-responsive metabolite in the subject to a reference level of the metabolite in subjects suffering from ASD and one or more gastrointestinal disorders, wherein substantial identity between the blood level of the metabolites in the subject and the reference level indicates that the subject is susceptible to the probiotic treatment.

[0021] In some embodiments, the method includes adjusting the composition of gut microbiota of the subject.

[0022] In some embodiments, adjusting the composition of gut microbiota of the subject comprises administering the subject a composition comprising *Bacteroides* bacteria. In some embodiments, the *Bacteroides* bacteria is *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, or a mixture thereof.

[0023] In some embodiments, adjusting the composition of gut microbiota of the subject comprises fecal transplantation.

[0024] In some embodiments, the *B. fragilis*-responsive metabolite is one of the metabolites listed in Table 2.

[0025] Some embodiments disclosed herein are related to a method for relieving gastrointestinal (GI) distress of a subject suffering from autism spectrum disorder (ASD), comprising reducing intestinal permeability in the subject. In some embodiments, the GI distress comprises abdominal cramps, chronic diarrhea, constipation, intestinal permeability, or a combination thereof. In some embodiments, reducing intestinal permeability comprises adjusting the composition of gut microbiota in the subject.

[0026] Also disclosed herein in some embodiments is a method for diagnosing autism spectrum disorder (ASD) in a subject, where the method includes: determining the level of a cytokine in gut and the blood level of one or more ASD-related metabolites in the subject; and detecting whether or not there is an alteration in the level of the cytokine in gut and the blood level of at least one or more of the ASD-related metabolites in the subject as compared to a reference level of the cytokine and the metabolite in non-autistic subjects, whereby an alteration in the amount of the cytokine in gut and the blood level of at least one of the one or more metabolites indicates that the subject suffers from ASD.

[0027] Further disclosed herein in some embodiments is a method for diagnosing autism spectrum disorder (ASD) in a subject, where the method includes: determining the blood level of two or more ASD-related metabolites in the subject; and detecting whether or not there is an alteration in the blood level of the two or more ASD-related metabolites in the subject as compared to a reference level of the metabolites in non-autistic subjects, whereby an alteration in the blood level of at least two of the two or more ASD-related metabolites indicates that the subject suffers from ASD.

[0028] In some embodiments, the one or more of the ASD-related metabolites are selected from the metabolites listed in Table 1. In some embodiments, the one or more ASD-related metabolites comprises a metabolite involved in tryptophan metabolism, a metabolite involved in fatty acid metabolism, a metabolite involved in purine metabolism, glycolate, imidazole propionate, N-acetylserine, or any combination thereof. In some embodiments, the metabolite involved in tryptophan metabolism is 4-ethylphenylsulfate, indolepyruvate,

indolyl-3-acryloylglycine, or serotonin. In some embodiments, the cytokine is interleukin-6 (IL-6). In some embodiments, the method includes altering the level of one or more ASD-related metabolites in the subject to improve behavioral performance in the subject if it is indicated that the subject suffers from ASD.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] **Fig. 1.** MIA offspring exhibit deficient GI barrier integrity and abnormal expression of tight junction components and cytokines. **Fig. 1A.** Intestinal permeability assay, measuring fluorescence intensity of fluorescein isothiocyanate (FITC) detected in serum after oral gavage of FITC-dextran. DSS: n=6, S: adult n=16; adolescent n=4, P: adult n=17; adolescent n=4. Data are normalized to fluorescence intensity observed in adult saline offspring. **Fig. 1B.** Expression of tight junction components relative to beta-actin in colons of adult saline and poly(I:C) offspring. Data for each gene are normalized to expression levels in saline offspring. n=8. **Fig. 1C.** Expression of cytokines and inflammatory markers relative to beta-actin in colons of adult saline and poly(I:C) offspring. Data for each gene are normalized to expression levels in saline offspring. n=6-21. **Fig. 1D.** Protein levels of cytokines and chemokines relative to total protein content in colons of adult saline and poly(I:C) offspring. n=10. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DSS=dextran sodium sulfate, S=saline+vehicle, P=poly(I:C) +vehicle. For each experiment, adult saline and poly(I:C) offspring were treated with vehicle at 3 weeks of age, and data were collected simultaneously for poly(I:C)+*B. fragilis* treatment group.

[0030] **Figure 2.** *B. fragilis* treatment has little effect on tight junction expression and cytokine profiles in the small intestine. **Fig. 2A.** Expression of tight junction components relative to beta-actin in small intestines of adult saline and poly(I:C) offspring. Data for each gene are normalized to expression levels in saline offspring. n=8. **Fig. 2B.** Quantification of the effect of *B. fragilis* treatment on expression of notable tight junction components relative to beta-actin in small intestines of MIA offspring. Data for saline and poly(I:C) are as in panel (A). n=8. **Fig. 2C.** Protein levels of cytokines and chemokines relative to total protein content in small intestines of adult saline, poly(I:C) and poly(I:C)+*B. fragilis* offspring. Data is normalized to expression levels in saline offspring. Asterisks directly above bars indicate significance compared to saline control (normalized to 1, as denoted by the black line),

whereas asterisks at the top of the graph denote statistical significance between poly(I:C) and poly(I:C)+*B. fragilis* groups. n=8-10. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis*

[0031] Figure 3. *B. fragilis* treatment has no effect on systemic immune dysfunction in MIA offspring. **Fig. 3A.** Percent frequency of Foxp3+ CD25+ T regulatory cells from a parent population of CD4+ TCRb+ cells, measured by flow cytometry of splenocytes from adult saline, poly(I:C) and poly(I:C)+*B. fragilis* offspring. n=5. **Fig. 3B.** Percent frequency of CD4+ T helper cells and CD11b+ and Gr-1+ neutrophilic and monocytic cells from a parent population of TER119- cells, measured by flow cytometry of splenocytes from adult saline, poly(I:C) and poly(I:C)+*B. fragilis* offspring. n=5. **Fig. 3C.** Production of IL-17 and IL-6 by splenic CD4+ T cells isolated from adult saline and poly(I:C) offspring, after *in vitro* stimulation with PMA/ionomycin. Treatment effects were assessed by repeated measures two-way ANOVA with Bonferroni post-hoc test. n=5. **Fig. 3D.** Production of IL-17 and IL-6 by CD4+ T cells isolated from mesenteric lymph nodes of adult saline and poly(I:C) offspring, after *in vitro* stimulation with PMA/ionomycin. Treatment effects were assessed by repeated measures two-way ANOVA with Bonferroni post-hoc test. n=5. **Fig. 3E.** Anxiety-like and locomotor behavior in the open field exploration assay for adult MIA offspring treated with mutant *B. fragilis* lacking production of polysaccharide A (PSA). Data indicate total distance traveled in the 50 x 50 cm open field (right), duration spent in the 17 x17 cm center square (middle) and number of entries into the center square (left) over a 10-minute trial. Data for saline, poly(I:C) and poly(I:C)+*B. fragilis* groups are as in Figure 10. poly(I:C)+*B. fragilis* with PSA deletion: n=17. **Fig. 3F.** Repetitive burying of marbles in a 6 x 8 array in a 10-minute trial. Data for saline, poly(I:C) and poly(I:C)+*B. fragilis* groups are as in Figure 10. poly(I:C)+*B. fragilis* with PSA deletion: n=17. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis,* P+BFΔPSA=poly(I:C)+*B. fragilis* with PSA deletion.

[0032] Figure 4. MIA induces alterations in the composition of the intestinal microbiota. **Fig. 4A.** Richness of the gut microbiota, as illustrated by rarefaction curves plotting Faith's Phylogenetic Diversity (PD) versus the number of sequences for each treatment group. **Fig. 4B.** Evenness of the gut microbiota, as indicated by the Gini

coefficient. **Fig. 4C.** Levels of *B. fragilis* 16S sequence (top) and bacterial 16S sequence (bottom) in fecal samples collected at 1, 2, and 3 weeks post treatment of adult offspring with vehicle or *B. fragilis*. Germ-free mice colonized with *B. fragilis* were used as a positive control. Data are presented as quantitative RT-PCR cycling thresholds [C(t)], where C(t)>34 (hatched line) is considered negligible, and for C(t)<34, lesser C(t) equates to stronger abundance. n=1, where each represents pooled sample from 3-5 independent cages. **Fig. 4D.** Levels of *B. fragilis* 16S sequence (top) and bacterial 16S sequence (bottom) in fecal samples collected at 1, 2, and 3 weeks post treatment of adult offspring with vehicle or *B. fragilis*. Germ-free mice colonized with *B. fragilis* were used as a positive control. Data are presented as quantitative RT-PCR cycling thresholds [C(t)], where C(t)>34 (hatched line) is considered negligible, and for C(t)<34, lesser C(t) equates to stronger abundance. n=1, where each represents pooled sample from 3-5 independent cages. Data are presented as mean ± SEM. S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis,* GF+BF=germ-free+*B. fragilis*.

[0033] **Figure 5.** MIA offspring exhibit dysbiosis of the intestinal microbiota. **Fig. 5A** is an unweighted UniFrac-based 3D PCoA plot based on all OTUs, illustrating global differences in the gut microbiota between adult MIA and control offspring. The percent variation explained by each principal coordinate (PC) is indicated on the axes. **Fig. 5B** is an unweighted UniFrac-based 3D PCoA plot based on subsampling of Clostridia and Bacteroidia OTUs (2003 reads per sample). **Fig. 5C** is an unweighted UniFrac-based 3D PCoA plot based on subsampling of OTUs remaining after subtraction of Clostridia and Bacteroidia OTUs (47 reads per sample). **Fig. 5D** is a heat-map showing the relative abundance of unique OTUs of the gut microbiota (bottom, x-axis) for individual biological replicates from adult saline and poly(I:C) offspring (right, y-axis), where red of increasing intensity denotes increasing relative abundance of a unique OTU for a particular sample. All OTUs that significantly discriminate between treatment groups are plotted. Family-level taxonomic assignments as designated by the Ribosomal Database Project are indicated for each OTU. **Fig. 5E** shows mean relative abundance of OTUs classified by taxonomic assignments at the class level for the most abundant taxa (left) and least abundant taxa (right). n=10. Data were simultaneously collected and analyzed for poly(I:C)+*B. fragilis* treatment group.

[0034] **Figure 6.** *B. fragilis* treatment corrects deficits in GI barrier integrity and colon expression of tight junction components and cytokines in MIA offspring. **Fig. 6A.** Intestinal permeability assay, measuring fluorescence intensity of fluorescein isothiocyanate (FITC) detected in serum after oral gavage of FITC-dextran. Data are normalized to fluorescence intensity observed in adult saline offspring. Data for DSS, saline and poly(I:C) are as in Figure 1. poly(I:C)+*B. fragilis*: n=9. **Fig. 6B.** Expression of tight junction components relative to beta-actin in colons of adult saline, poly(I:C) and poly(I:C)+*B. fragilis* offspring. Data for each gene are normalized to expression levels in saline offspring. Data for saline and poly(I:C) are as in Figure 1. Asterisks directly above bars indicate significance compared to saline control (normalized to 1, as denoted by the black line), whereas asterisks at the top of the graph denote statistical significance between poly(I:C) and poly(I:C)+*B. fragilis* groups. n=8. **Fig. 6C.** Immunofluorescence staining for claudin 8. Representative images for n=5. **Fig. 6D.** Protein levels of claudin 8 (left) and claudin 15 (right) in colons from saline, poly(I:C) and poly(I:C)+*B. fragilis* offspring, as measured by Western blot. Representative signals from the same blot are depicted below. Data are normalized to signal intensity detected in saline offspring. n=3. **Fig. 6E.** Expression of IL-6 relative to beta-actin in colons of adult saline, poly(I:C) and poly(I:C)+*B. fragilis* offspring. Data is normalized to expression levels in saline offspring. Data for saline and poly(I:C) are as in Figure 1. poly(I:C)+*B. fragilis*: n=3. **Fig. 6F.** Protein levels of cytokines and chemokines relative to total protein content in colons of adult saline, poly(I:C) and poly(I:C)+*B. fragilis* offspring. Data is normalized to expression levels in saline offspring. Data for saline and poly(I:C) are as in Figure 1. Asterisks directly above bars indicate significance compared to saline control (normalized to 1, as denoted by the black line), whereas asterisks at the top of the graph denote statistical significance between poly(I:C) and poly(I:C)+B. fragilis groups. n=10. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, n.s.=not significant. DSS=dextran sodium sulfate, S=saline+vehicle, P=poly(I:C) +vehicle, P+BF=poly(I:C)+*B. fragilis*.

[0035] **Figure 7.** IL-6 modulates colon expression of claudin 8 and 15. **Fig. 7A.** Dose-dependent expression of claudin 8 (left) and claudin 15 (right) relative to beta-actin in colons of adult wild-type mice cultured for 4 hours *ex vivo* with increasing concentrations of recombinant mouse IL-6. Data are normalized to expression levels detected in 0 ng/ml IL-6

controls. n=3. **Fig. 7B.** Time-dependent expression of claudin 8 (left) and claudin 15 (right) relative to beta-actin in colons of adult wild-type mice cultured with 80 ng/ml recombinant mouse IL-6. n=3. **Fig. 7C.** Expression of claudin 8 (top) and claudin 15 (bottom) relative to beta-actin in colons of adult wild-type mice at 12 hours post treatment with recombinant mouse IL-6. n=3. Data are presented as mean ± SEM.

[0036] **Figure 8.** *B. fragilis* treatment alters the composition of the intestinal microbiota and corrects species-level abnormalities in MIA offspring. **Fig. 8A** is an unweighted UniFrac-based 3D PCoA plot based on all OTUs. The percent variation explained by each principal coordinate (PC) is indicated on the axes. Data for saline and poly(I:C) are as in Figure 2. **Fig. 8B.** Relative abundance of key OTUs of the family Lachnospiraceae (top) and order Bacteroidales (bottom) that are significantly altered by MIA and completely restored by *B. fragilis* treatment. Data are presented as mean ± SEM. **Fig. 8C** is a phylogenetic tree based on nearest-neighbor analysis of 16S rRNA gene sequences for key OTUs presented in panel B. Clades shown in solid lines indicate OTUs of the family Lachnospiraceae and clades showing in broken lines indicate OTUs of the order Bacteriodales. The 6 taxa labeled with numbers indicate OTUs that are significantly elevated in poly(I:C) offspring and corrected by *B. fragilis* treatment. n=10.

[0037] **Figure 9.** There is no evidence for persistent colonization of *B. fragilis* after treatment of MIA offspring. **Fig. 9A.** Levels of *B. fragilis* 16S sequence (top) and bacterial 16S sequence (bottom) in fecal samples collected at 1, 2, and 3 weeks post treatment of adult offspring with vehicle or *B. fragilis*. Germ-free mice colonized with *B. fragilis* were used as a positive control. Data are presented as quantitative RT-PCR cycling thresholds [C(t)], where C(t)>34 (hatched line) is considered negligible, and for C(t)<34, lesser C(t) equates to stronger abundance. n=1, where each represents pooled sample from 3-5 independent cages. **Fig. 9B.** Levels of *B. fragilis* 16S sequence (top) and bacterial 16S sequence (bottom) in fecal samples collected at 1, 2, and 3 weeks post treatment of adult offspring with vehicle or *B. fragilis*. Germ-free mice colonized with *B. fragilis* were used as a positive control. Data are presented as quantitative RT-PCR cycling thresholds [C(t)], where C(t)>34 (hatched line) is considered negligible, and for C(t)<34, lesser C(t) equates to stronger abundance. n=1, where each represents pooled sample from 3-5 independent cages.

Data are presented as mean ± SEM. S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis,* GF+BF=germ-free+*B. fragilis*.

[0038] Figure 10. *B. fragilis* treatment ameliorates autism-related behavioral abnormalities in MIA offspring. **Fig. 10A.** Anxiety-like and locomotor behavior in the open field exploration assay, as measured by total distance traveled in the 50 x 50 cm open field (right), duration spent in the 17 x17 cm center square (middle), and number of entries into the center of the field (left) over a 10 minute trial. n=35-75. **Fig. 10B.** Sensorimotor gating in the pre-pulse inhibition assay, as measured by percent difference between the startle response to pulse only and startle response to pulse preceded by a 5 db or 15 db pre-pulse. Treatment effects were assessed by repeated measures two-way ANOVA with Bonferroni post-hoc test. n=35-75. **Fig. 10C.** Repetitive burying of marbles in a 3 x 6 array during a 10 minute trial. n=16-45. **Fig. 10D.** Communicative behavior, as measured by total number (left), average duration (middle) and total duration (right) of ultrasonic vocalizations produced by adult male mice during a 10 minute social encounter. n=10. **Fig. 10E** shows deficits in sociability in *B. fragilis*-treated MIA offspring. **Fig. 10F** shows deficits in social preference in *B. fragilis*-treated MIA offspring. Graphs represent cumulative results obtained for 3-6 independent cohorts of mice. Data are presented as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis.* Data were collected simultaneously for poly(I:C)+*B. fragilis* ΔPSA and poly(I:C)+*B. thetaiotaomicron* treatment groups.

[0039] Figure 11. Amelioration of autism-related behaviors in MIA offspring is not specific to *B. fragilis* treatment. **Fig. 11A.** Anxiety-like and locomotor behavior in the open field exploration assay, as measured by total distance traveled in the 50 x 50 cm open field (right), duration spent in the 17 x17 cm center square (middle), and number of entries into the center of the field (left) over a 10 minute trial. Poly(I:C)+*B. thetaiotaomicron*: n=32. **Fig. 11B.** Repetitive burying of marbles in a 3 x 6 array during a 10 minute trial. Poly(I:C)+*B. thetaiotaomicron*: n=32. **Fig. 11C.** Communicative behavior, as measured by total number (left), average duration (middle) and total duration (right) of ultrasonic vocalizations produced by adult male mice during a 10 minute social encounter. Poly(I:C)+*B. thetaiotaomicron*: n=10. **Fig. 11D.** Sensorimotor gating in the pre-pulse inhibition assay, as measured by percent difference between the startle response to pulse only and startle

response to pulse preceded by a 5 db or 15 db pre-pulse. Treatment effects were assessed by repeated measures two-way ANOVA with Bonferroni post-hoc test. Poly(I:C)+*B. thetaiotaomicron*: n=32. For all panels, data for saline, poly(I:C) and poly(I:C)+*B. fragilis* are as in Figure 10. Graphs represent cumulative results obtained for 3-6 independent cohorts of mice. Data are presented as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis,* P+BT=Poly(I:C)+*B. thetaiotaomicron.*

[0040] Figures 12A-B. *B. fragilis* treatment causes statistically significant alterations serum metabolites, with widespread changes in biochemicals relevant to fatty acid metabolism and purine salvage pathways. Levels of 103 metabolites that are significantly altered in sera of *B. fragilis*-treated MIA offspring compared to saline controls, as measured by GC/LC-MS. Colors indicate fold change relative to metabolite concentrations detected in saline offspring, where red hues represent increased levels compared to controls and green hues represent decreased levels compared to controls (see legend on top left). All changes indicated are $p<0.05$ by two-way ANOVA with contrasts. P=poly(I:C), P+BF=poly(I:C)+*B. fragilis.* n=8.

[0041] Figure 13. *B. fragilis* treatment corrects MIA-induced alterations in 4-ethylphenylsulfate (4EPS), a microbe-dependent metabolite that sufficiently induces anxiety-like behavior. **Fig. 13A** shows relative quantification of metabolites detected by GC/LC-MS that were significantly altered by MIA and restored by *B. fragilis* treatment. n=8. **Fig. 13B** shows concentrations of 4EPS detected by LC-MS in sera of adult germ-free (GF) versus conventionally-colonized (specific pathogen-free, SPF) mice. U.D.=undetectable. n=1, where each represents pooled sera from 3-5 mice. **Fig. 13C.** Anxiety-like and locomotor behavior in the open field exploration assay for conventional wild-type mice treated with 4EPS or saline vehicle. Data indicate total distance traveled in the 50 x 50 cm open field (right) and duration spent in the 17 x17 cm center square (left) over a 10 minute trial. There is no difference between 4EPS- and vehicle-treated mice in number of entries into the center of the field (data not shown). n=10. **Fig. 13D.** Potentiated startle reflex in the pre-pulse inhibition assay in 4EPS-treated mice compared to controls. Data show the average intensity of startle in response to a 120 db pulse (left) and percent inhibition of the pulse when it is preceded by a 5 db or 15 db pre-pulse (right). n=10**.** Data are presented as mean ± SEM. $*p < 0.05$, $**p <$

0.01, S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis,* SPF=specific pathogen-free (conventionally-colonized), GF=germ-free, Veh.=vehicle (saline), 4EPS=4-ethylphenylsulfate.

[0042] **Figure 14.** Synthesis of autism-associated metabolites by host-microbe interactions. **Fig. 14A.** Diagram illustrating the synthesis of 4EPS (found elevated in MIA serum and restored by *B. fragilis* treatment) and *p*-cresol (reported to be elevated in urine of individuals with ASD) by microbial tyrosine metabolism and host sulfation. **Fig. 14B.** Diagram illustrating the synthesis of indolepyruvate (found elevated in MIA serum and restored by *B. fragilis* treatment) and indolyl-3-acryloylglycine (reported to be elevated in urine of individuals with ASD) from microbial tryptophan metabolism and host glycine conjugation. Solid arrows represent known biological conversions. Dotted arrow represents predicted biological conversions.

[0043] **Figure 15.** 4-ethylphenylsulfate (4EPS) synthesis, detection and *in vivo* experiments. **Fig. 15A.** Diagram of 4EPS synthesis by treating 4-ethylphenol with sulfur trioxide-pyridine in refluxing benzene to generate the pyridinium salt followed by ion exchange over K+ resin to generate the potassium salt. **Fig. 15B.** Dose-response curve and linear regression analysis for known concentrations of potassium 4EPS analyzed by LC/MS. **Fig. 15C.** Time-dependent increases in serum 4EPS after a single i.p. injection of 30 mg/kg potassium 4EPS into adult wild-type mice. **Fig. 15D.** Communicative behavior, as measured by total number (left), average duration (middle) and total duration (right) of ultrasonic vocalizations produced by adult male mice during a 10-minute social encounter. n=5. **Fig. 15E.** Repetitive burying of marbles in a 3 x 6 array during a 10-minute trial. n=10. Data are presented as mean ± SEM. Veh.=vehicle (saline), 4EPS=4-ethylphenylsulfate.

DETAILED DESCRIPTION

[0044] In the following detailed description, reference is made to the accompanying drawings, which form a part hereof. In the drawings, similar symbols typically identify similar components, unless context dictates otherwise. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. Other embodiments may be utilized, and other changes may be made, without departing from the spirit or scope of the subject matter presented herein. It will be readily

understood that the aspects of the present disclosure, as generally described herein, and illustrated in the Figures, can be arranged, substituted, combined, separated, and designed in a wide variety of different configurations, all of which are explicitly contemplated herein.

[0045] Autism spectrum disorder (ASD) is a serious neurodevelopmental disorder characterized by stereotypic behaviors and deficits in language and social interaction. As described herein, various metabolites are related to ASD. The level of these metabolites in a subject can be determined and used to diagnose ASD, or adjusted for treating ASD, such as by improving behavioral performance of the subject. In addition, as described herein, various metabolites are responsive to *B. fragilis* treatment, and those metabolites can be used to assess the susceptibility of a subject suffering from ASD to probiotic treatment.

[0046] In some embodiments, the level of the metabolite in the circulation of a subject in need of treatment is determined and adjusted to improve behavioral performance in the subject. The subject in need of treatment can be a subject suffering from anxiety, ASD, or a pathological condition with one or more of the symptoms of ASD. The level of the metabolite in the circulation of the subject can be the blood level, for example the serum level or plasma level, of the metabolite. In some embodiments, the urine or fecal level of the metabolite in the subject is determined and adjusted to improve behavioral performance in the subject.

[0047] In some embodiments, the level of the metabolite in the circulation of a subject is detected and compared with a reference level of the metabolite in non-autistic population to diagnose whether the subject has ASD or not. The level of the metabolite in the circulation of the subject can be the blood level, for example the serum level or plasma level, of the metabolite.

Definitions

[0048] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present disclosure belongs. *See, e.g.* Singleton et al., Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, NY 1994); Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press (Cold Springs Harbor, NY 1989). For purposes of the present disclosure, the following terms are defined below.

[0049] As used herein, the term "subject" is a vertebrate, such as a mammal. The term "mammal" is defined as an individual belonging to the class Mammalia and includes, without limitation, humans, domestic and farm animals, and zoo, sports, or pet animals, such as sheep, dogs, horses, cats or cows. In some embodiments, the subject is human.

[0050] As used herein, the term "condition/disorder/symptom" or "behavioral abnormality" refers to a symptom expressed by a subject including but not limited to anxiety, Fragile X, Rett syndrome, tuberous sclerosis, obsessive compulsive disorder, attention deficit disorder, schizophrenia, autistic disorder (classic autism), Asperger's disorder (Asperger syndrome), pervasive developmental disorder not otherwise specified (PDD-NOS), childhood disintegrative disorder (CDD), or a pathological condition with one or more of the symptoms of ASD.

[0051] As used herein, the term "subject in need of the treatment" refers to a subject expressing or suffering from one or more of the behavioral disorder/symptoms mentioned above. An appropriately qualified person is able to identify such an individual in need of treatment using standard behavioral testing protocols/guidelines. The same behavioral testing protocols/guidelines can also be used to determine whether there is improvement to the individual's disorder and/or symptoms.

[0052] As used herein, the term "improvement in behavioral performance" refers prevention or reduction in the severity or frequency, to whatever extent, of one or more of the behavioral disorders, symptoms and/or abnormalities expressed by individual suffering from anxiety, ASD, or a pathological condition with one or more of the symptoms of ASD. Non-limiting examples of the behavioral symptom include repetitive behavior, decreased prepulse inhibition (PPI), and increased anxiety. The improvement is either observed by the individual taking the treatment themselves or by another person (medical or otherwise).

[0053] As used herein, the term "treatment" refers to a clinical intervention made in response to a disease, disorder or physiological condition manifested by a patient, particularly a patient suffering from ASD. The aim of treatment may include, but is not limited to, one or more of the alleviation or prevention of symptoms, slowing or stopping the progression or worsening of a disease, disorder, or condition and the remission of the disease, disorder or condition. In some embodiments, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include

those already affected by a disease or disorder or undesired physiological condition as well as those in which the disease or disorder or undesired physiological condition is to be prevented. For example, in some embodiments treatment may improve behavioral performance of the subject, including ASD-related behaviors. As used herein, the term "prevention" refers to any activity that reduces the burden of the individual later expressing those behavioral symptoms. This takes place at primary, secondary and tertiary prevention levels, wherein: a) primary prevention avoids the development of symptoms/disorder/condition; b) secondary prevention activities are aimed at early stages of the condition/disorder/symptom treatment, thereby increasing opportunities for interventions to prevent progression of the condition/disorder/symptom and emergence of symptoms; and c) tertiary prevention reduces the negative impact of an already established condition/disorder/symptom by, for example, restoring function and/or reducing any condition/disorder/symptom or related complications.

[0054] "Pharmaceutically acceptable" carriers are ones which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. "Pharmaceutically acceptable" carriers can be, but not limited to, organic or inorganic, solid or liquid excipents which is suitable for the selected mode of application such as oral application or injection, and administered in the form of a conventional pharmaceutical preparation, such as solid such as tablets, granules, powders, capsules, and liquid such as solution, emulsion, suspension and the like. Often the physiologically acceptable carrier is an aqueous pH buffered solution such as phosphate buffer or citrate buffer. The physiologically acceptable carrier may also comprise one or more of the following: antioxidants including ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, such as serum albumin, gelatin, immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids, carbohydrates including glucose, mannose, or dextrins, chelating agents such as EDTA, sugar alcohols such as mannitol or sorbitol, salt-forming counterions such as sodium, and nonionic surfactants such as Tween™, polyethylene glycol (PEG), and Pluronics™. Auxiliary, stabilizer, emulsifier, lubricant, binder, pH adjustor controller, isotonic agent and other conventional additives may also be added to the carriers.

[0055] The pharmaceutically acceptable or appropriate carrier may include other compounds known to be beneficial to an impaired situation of the GI tract, (e.g.,

antioxidants, such as Vitamin C, Vitamin E, Selenium or Zinc); or a food composition. The food composition can be, but is not limited to, milk, yoghurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacterial suspensions, dried oral supplement, or wet oral supplement.

[0056] As used herein, the term "neutraceutical" refers to a food stuff (as a fortified food or a dietary supplement) that provides health benefits. Nutraceutical foods are not subject to the same testing and regulations as pharmaceutical drugs.

[0057] As used herein, the term "probiotic" refers to live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host. The probiotics may be available in foods and dietary supplements (for example, but not limited to capsules, tablets, and powders). Non-limiting examples of foods containing probiotic include dairy products such as yogurt, fermented and unfermented milk, smoothies, butter, cream, hummus, kombucha, salad dressing, miso, tempeh, nutrition bars, and some juices and soy beverages.

[0058] As used herein, the term "metabolite" refers to any molecule involved in metabolism. Metabolites can be products, substrates, or intermediates in metabolic processes. For example, the metabolite can be a primary metabolite, a secondary metabolite, an organic metabolite, or an inorganic metabolite. Metabolites include, without limitation, amino acids, peptides, acylcarnitines, monosaccharides, lipids and phospholipids, prostaglandins, hydroxyeicosatetraenoic acids, hydroxyoctadecadienoic acids, steroids, bile acids, and glycolipids and phospholipids.

[0059] As used herein, the term "cytokine" refers to a secreted protein or active fragment or mutant thereof that modulates the activity of cells of the immune system. Examples of cytokines include, without limitation, interleukins, interferons, chemokines, tumor necrosis factors, colony-stimulating factors for immune cell precursors, and the like.

[0060] As used herein, the term "antibody" includes polyclonal antibodies, monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, multispecific antibodies (e.g., bispecific antibodies, diabodies, and single-chain molecules, and antibody fragments (e.g., Fab or $F(ab')_2$, and Fv). For the structure and properties of the different classes of antibodies,

see e.g., Basic and Clinical Immunology, 8th Edition, Daniel P. Sties, Abba I. Terr and Tristram G. Parsolw (eds), Appleton & Lange, Norwalk, Conn., 1994, page 71 and Chapter 6.

Autism spectrum disorder (ASD)

[0061] Autism spectrum disorders (ASDs) are complex neurodevelopmental disabilities characterized by stereotypic behaviors and deficits in communication and social interaction. The term "spectrum" refers to the wide range of symptoms, skills, and levels of impairment, or disability, that patients with ASD can have. ASD is generally diagnosed according to guidelines listed in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition – Text Revision (DSM-IV-TR). The manual currently defines five disorders, sometimes called pervasive developmental disorders (PDDs), as ASD, including Autistic disorder (classic autism), Asperger's disorder (Asperger syndrome), Pervasive developmental disorder not otherwise specified (PDD-NOS), Rett's disorder (Rett syndrome), and Childhood disintegrative disorder (CDD). Some patients are mildly impaired by their symptoms, but others are severely disabled. ASD encompasses a set of complex disorders with poorly defined etiologies, and no targeted cure

[0062] Recent studies highlight striking neural and peripheral immune dysregulation in autistic individuals. Among several comorbidities in ASD, gastrointestinal (GI) distress is of particular interest, given its prevalence and correlation with the severity of core autism behaviors (Adams et al., 2011; Buie et al., 2010; Coury et al., 2012; Gorrindo et al., 2013; Ibrahim et al., 2009; Wang et al., 2011). A significant subset of ASD children exhibit gastrointestinal (GI) complications, including increased intestinal permeability (or "leaky gut" and altered composition of intestinal microbiota (Buie et al., 2010; Coury et al., 2012; D'Eufemia et al., 1996; de Magistris et al., 2010; de Magistris et al., 2013; Ibrahim et al., 2009). Moreover, a recent multicenter study of over 14,000 ASD individuals reports a higher prevalence of inflammatory bowel disease (IBD) and other GI disorders in ASD patients compared to controls (Kohane et al., 2012). Altered nutrient intake, food allergies and metabolic disruptions are also associated with ASD, and antibiotic treatment and restricted diet are reported to provide behavioral improvements for some autistic children (Buie et al., 2010).

[0063] Maternal immune activation (MIA) is an important environmental risk factor for ASD. Several large epidemiological studies have linked maternal viral and bacterial infection with increased autism risk in the offspring ((Atladottir et al., 2010; Gorrindo et al., 2012). Modeling this risk factor in mice by injecting pregnant females with the viral mimic poly(I:C) has been show to yield offspring that exhibit the core behavioral symptoms of autism, including the hallmark symptoms of repetitive/compulsive behaviors, as well as a common autism neuropathology (spatially restricted deficits in Purkinje cells) ((Boksa, 2010; Malkova et al., 2012; Schwartzer et al., 2013; Shi et al., 2009). Recently, MIA offspring have also been found to exhibit abnormalities in the immune system and the gastrointestinal tract.

[0064] Humans are colonized with a great abundance and diversity of microbes, which play a critical role in regulating health and disease. Dysbiosis of the commensal microbiota is implicated in the pathogenesis of several human ailments, including IBD, obesity and cardiovascular disease (Blumberg and Powrie, 2012; Clemente et al., 2012). Commensal bacteria also affect a variety of complex behaviors, including social, emotional, nociceptive and anxiety-like behaviors (Amaral et al., 2008; Bravo et al., 2011; Desbonnet et al., 2013; Heijtz et al., 2011), and contribute to brain development and function in mice (Al-Asmakh et al., 2012; Collins et al., 2012; Cryan and Dinan, 2012) and humans (Tillisch et al., 2013). Long-range interactions between the gut microbiota and brain underlie the ability of microbe-based therapies to treat symptoms of multiple sclerosis and depression in mice (Bravo et al., 2011; Hooper et al., 2012; Ochoa-Reparaz et al., 2010) and the reported efficacy of probiotics in treating emotional symptoms of chronic fatigue syndrome and psychological distress in humans (Messaoudi et al., 2011; Rao et al., 2009).

[0065] Numerous abnormalities related to the microbiota have been identified in autistic individuals, including disrupted community composition (Adams et al., 2011; Finegold, 2011; Finegold et al., 2010; Finegold et al., 2012; Gondalia et al., 2012; Parracho et al., 2005b; Williams et al., 2011; Williams et al., 2012) and altered peripheral levels of microbially-derived metabolites (Altieri et al., 2011; Frye et al., 2013; MacFabe, 2012; Ming et al., 2012b; Yap et al., 2010a).

Methods for improving behavioral performance

[0066] Methods for improving behavioral performance in a subject in need of treatment are provided herein. The subject in need of treatment can be a subject suffering from anxiety, ASD, or a pathological condition with one or more of the symptoms of ASD.

[0067] The methods, in some embodiments, include: determining the blood level of an ASD-related metabolite in a subject in need of treatment; and adjusting the blood level of the ASD-related metabolite in the subject until an improvement in the behavioral performance in the subject is observed.

[0068] The methods, in some embodiments, include: determining the level of an autism spectrum disorder (ASD)-related metabolite in a subject in need of treatment; and adjusting the level of the ASD-related metabolite in the subject so that the level of the metabolite in the subject is substantially the same as a reference level of the metabolite in non-autistic subjects, thereby improving behavioral performance in the subject. In some embodiments, the methods can further include determining a reference level of the ASD-related metabolite in a population of non-autistic subjects.

[0069] In some embodiments, the methods include: determining the level of an autism spectrum disorder (ASD)-related metabolite in a subject in need of treatment; and adjusting the level of the ASD-related metabolite in the subject so that the level of the metabolite in the subject is substantially the same as a reference level of the metabolite in a population of subjects that do not suffer ASD, anxiety or any pathological condition with one or more of the symptoms of ASD, thereby improving behavioral performance in the subject. In some embodiments, the methods can further include determining a reference level of the ASD-related metabolite in subjects that do not suffer from ASD, anxiety or any pathological condition with one or more of the symptoms of ASD.

[0070] The methods disclosed herein, in some embodiments, can also include measuring a baseline of behavioral performance prior to adjusting the level of the ASD-related metabolite in the subject in need of treatment and/or measuring the behavioral performance after adjusting the level of the ASD-related metabolite in the subject in need of treatment. In some embodiments, the methods can include comparing the behavioral performance prior to and after adjusting the level of the ASD-related metabolite in the

subject in need of treatment, and the comparison can be used to determine if and to what extent the behavioral performance in the subject is improved.

[0071] In the method disclosed herein, behavioral performance can be measured and evaluated using various parameters and methods. For example, behavioral test can be conducted to determine the presence and/or extent of restricted repetitive behavior and/or stereotyped behavior patterns of the subject under test. In some embodiments, the Autism Behavior Checklist (ABC), Autism diagnostic Interview-Revised (ADI-R), childhood autism Rating Scale (CARS), and/or Pre-Linguistic Autism Diagnostic Observation Schedule (PL-ADOS) is used for the behavioral test. The behavioral test can include, but is not limited to, detecting the presence and/or extent of 1) preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal in either intensity or focus, 2) inflexible adherence to specific, nonfunctional routines or rituals, c) stereotyped and repetitive motor mannerisms (such as hand flapping, finger flapping etc.), and/or d) persistent preoccupation with parts of objects. Non-limiting examples of behavior that can be included in a behavioral test and suggest a need for improving behavioral performance in the subject under the test include: a) sensory behaviors, including poor use of visual discrimination when learning, seems not to hear, so that a hearing loss is suspected, sometimes shows no "startle response" to loud noise", sometimes painful stimuli such as bruises, cuts, and injections evoke no reaction, often will not blink when bright light is directed toward eyes, covers ears at many sounds, squints, frowns, or covers eyes when in the presence of natural light, frequently has no visual reaction to a "new" person, stares into space for long periods of time; b) relating behaviors: frequently does not attend to social/environmental stimuli, has no social smile, does not reach out when reached for, non-responsive to other people's facial expressions/feelings, actively avoids eye contact, resists being touched or held, is flaccid when held in arms, is stiff and hard to held, does not imitate other children at play, has not developed any friendships, often frightened or very anxious, "looks through" people; c) body and object use behaviors: whirls self for long periods of time, does not use toys appropriately, insists on keeping certain objects with him/her, rocks self for long periods of time, does a lot of lunging and darting, flaps hands, walks on toes, hurts self by banging head, biting hand, etc…, twirls, spins, and bangs objects a lot, will feel, smell, and/or taste objects in the environment, gets involved in complicated "rituals" such as lining things up,

etc…, is very destructive; and d) language behaviors: does not follow simple commands given once, has pronoun reversal, speech is atonal, does not respond to own name when called out among two others, seldom says "yes" or "I", does not follow simple commands involving prepositions, gets desired objects by gesturing, repeats phrases over and over, cannot point to more than five named objects, uses 0-5 spontaneous words per day to communicate wants and needs, repeats sounds or words over and over, echoes questions or statements made by others, uses at least 15 but less than 30 spontaneous phrases daily to communicate, learns a simple task but "forgets" quickly, strong reactions to changes in routine/environment, has "special abilities" in one area of development, which seems to rule out mental retardation, severe temper tantrums and/or frequent minor tantrums, hurts others by biting, hitting, kicking, etc…, does not wait for needs to be met, difficulties with toileting, does not dress self without frequent help, frequently unaware of surroundings, and may be oblivious to dangerous situations, prefers to manipulate and be occupied with inanimate things, and A developmental delay was identified at or before 30 months of age. One of ordinary skill in the art would appreciate that the attending physician would know how to identify a subject in need of treatment disclosed herein.

[0072] After adjustment, the level of the ASD-related metabolite in the subject can about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98%, about 99%, about 100%, about 101%, about 102%, about 105%, about 110%, about 120%, about 130%, about 140%, about 150%, or a range between any two of these values of the reference level of the metabolite in non-autistic subjects. In some embodiments, the level of the ASD-related metabolite in the subject is about 80%, about 90%, about 95%, about 98%, about 99%, about 100%, about 101%, about 102%, about 105%, about 110%, about 120%, or a range between any two of these values of the reference level of the metabolite in non-autistic subjects. In some embodiments, the level of the ASD-related metabolite in the subject is about 95%, about 98%, about 99%, about 100%, about 101%, about 102%, about 105%, or a range between any two of these values of the reference level of the metabolite in non-autistic subjects. The level of the metabolite can be the level of the metabolite in circulation of the subject. For example, the level of the metabolite can be the level of the metabolite in blood or other body fluids (e.g., cerebrospinal fluid, pleural fluid, amniotic fluid, semen, or saliva) of the subject. In some embodiments, the level of the metabolite is

the blood level of the metabolite in the subject. The blood level of the metabolite can be, for example, serum level or plasma level of the metabolite. In some embodiments, the level of the metabolite is the urine level of the metabolite in the subject.

[0073] In some embodiments, the subject suffers from anxiety, ASD, or a pathological condition with one or more of the symptoms of ASD. Non-limiting examples of ASD include Autistic disorder (classic autism), Asperger's disorder (Asperger syndrome), Pervasive developmental disorder not otherwise specified (PDD-NOS), Rett's disorder (Rett syndrome), and Childhood disintegrative disorder (CDD). In some embodiments, the subject suffers from ASD. In some embodiments, the subject suffers from autism.

[0074] Various methods can be used to adjust the level, for example blood level, of the ASD-related metabolite in the subject. In some embodiments, the level, for example blood level, of the metabolite is adjusted by adjusting the composition of gut microbiota in the subject. Adjustment of the composition of gut microbiota in the subject can be achieved by, for example, fecal transplantation (also known as fecal microbiota transplantation (FMT), fecal bacteriotherapy or stool transplant). Fecal transplantation can include a process of transplantation of fecal bacteria from a healthy donor, for example a non-autistic subject, to a recipient (e.g., a subject suffering from autism). The procedure of fecal transplantation can include single or multiple infusions (e.g., by enema) of bacterial fecal flora from the donor to the recipient.

[0075] In some embodiments, adjusting the composition of gut microbiota in the subject includes administering the subject a composition comprising bacteria, for example, a composition comprising *Bacteroides* bacteria. The *Bacteroides* bacteria that can be used in the method disclosed herein is not particularly limited. In some embodiments, the *Bacteroides* bacteria comprise *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, or a mixture thereof. In some embodiments, the *Bacteroides* bacteria can be *B. fragilis*. The composition comprising bacteria, for example a composition comprising *Bacteroides* bacteria, can be administered to the subject via various routes. For example, the composition can be administered to the subject via oral administration, rectum administration, transdermal administration, intranasal administration or inhalation. In some embodiments, the composition is administered to the subject orally. The composition comprising bacteria, such as *Bacteroides* bacteria, can also be in various forms. For example, the composition can be a

probiotic composition, a neutraceutical, a pharmaceutical composition, or a mixture thereof. In some embodiments, the composition is a probiotic composition. Each dosage for human and animal subjects preferably contains a predetermined quantity of the bacteria calculated in an amount sufficient to produce the desired effect. The actual dosage forms will depend on the particular bacteria employed and the effect to be achieved. The composition comprising bacteria, for example, a composition comprising *Bacteroides* bacteria, can be administered alone or in combination with one or more additional probiotic, neutraceutical, or therapeutic agents. Administration "in combination with" one or more further additional probiotic, neutraceutical, or therapeutic agents includes both simultaneous (at the same time) and consecutive administration in any order. Administration can be chronic or intermittent, as deemed appropriate by the supervising practitioner, particularly in view of any change in the disease state or any undesirable side effects. "Chronic" administration refers to administration of the composition in a continuous manner while "intermittent" administration refers to treatment that is done with interruption.

[0076] In some embodiments, adjusting the composition of gut microbiota in the subject includes reducing the level of one or more bacterial species in the subject. For example, the level of *Clostridia* bacteria (such as Lachnospiraceae) in the subject can be reduced to adjust the composition of gut microbiota in the subject. In some embodiments, the Lachnospiraceae is *Roseburia*. The level of Bacterioidia bacteria (such as Bacteroidales S24-7) can also be reduced to adjust the composition of gut microbiota in the subject. In some embodiments, the *Clostridia* bacteria is *Lachnospiraceae*. Various methods can be used to reduce the level of one or more bacteria species in the subject. For example, a reduced carbohydrate diet can be provided to the subject to reduce one or more intestinal bacterial species. Without being bound to any specific theory, it is believed that a reduced carbohydrate diet can restrict the available material necessary for bacterial fermentation to reduce intestinal bacterial species.

[0077] In some embodiments, adjusting the composition of gut microbiota in the subject includes increasing the level of one or more bacterial species in the subject. For example, the level of *Ruminococcaceae*, *Erysipelotrichaceae*, and/or *Alcaligenaceae* bacteria in the subject can be increased to adjust the composition of gut microbiota in the subject.

ASD-related metabolites

[0078] As used herein, the term "autism spectrum disorder (ASD)-related metabolite" refers to a metabolite whose level is altered in a subject suffering from ASD, anxiety, and/or any pathological condition with one or more of the symptoms of ASD as compared to a non-autistic subject and/or a subject that does not suffer from ASD, anxiety or any pathological condition with one or more of the symptoms of ASD. For example, the level of the metabolite may be altered in circulation of the subject suffering from ASD as compared to a non-autistic subject. In some embodiments, the level of the metabolite is altered in blood, serum, plasma, body fluids (e.g., cerebrospinal fluid, pleural fluid, amniotic fluid, semen, or saliva), urine, and/or feces of the subject suffering from ASD as compared to a non-autistic subject. In some instances, the ASD-related metabolite plays a causative role in the development of ASD-related behaviors in the subject suffering from ASD. In some instances, the alteration in the level of ASD-related metabolite is caused by ASD. The ASD-related metabolite can have an increased or decreased level in the subject suffering from ASD as compared to a non-autistic subject or a subject that does not suffer from ASD, anxiety or any pathological condition with one or more of the symptoms of ASD.

[0079] One of ordinary skill in the art will appreciate that variability in the level of metabolites may exist between individuals, and a reference level can be established as a value representative of the level of the metabolites in a non-autistic population, or a population of subjects that do not suffer from ASD, anxiety or any pathological condition with one or more of the symptoms of ASD, for the comparison. Various criteria can be used to determine the inclusion and/or exclusion of a particular subject in the reference population, including age of the subject (e.g. the reference subject can be within the same age group as the subject in need of treatment) and gender of the subject (e.g. the reference subject can be the same gender as the subject in need of treatment). In some embodiments, the ASD-related metabolite has an increased level in the subject suffering from ASD as compared to the reference level. In some embodiments, the ASD-related metabolite has a decreased level in the subject suffering from ASD as compared to the reference level. In some embodiments, the alteration in the level of ASD-related metabolite can be restored partially or fully by adjusting the composition of gut microbiota in the subject suffering from ASD.

[0080] Non-limiting examples of ASD-related metabolites are provided in Table 1.

Table 1. Exemplary ASD-related metabolites

N-acetylserine	beta-alanine	4-methyl-2-oxopentaoate
imidazole propionate	phenol sulfate	5-methylthioadenosine
serotonin	3-methyl-2-oxovalerate	docosapentaenoate (n3 DPA; 22:5n3)
arginine	ornithine	docosapentaenoate (n6 DPA; 22:5n6)
glycylvaline	eicosenoate	dihomo-linoleate (20:2n6)
xylose	octadecanedioate	docosahexaenoate (DHA; 22:6n3)
stearate	pantothenate	1-pentadecanoylglycerophosphocholine
13-HODE+9-HODE	ergothioneine	1-oleoylglycerophosphoethanolamine
bilirubin (E,E)	glycolate (hydroxyacetate)	4-ethylphenylsulfate
equol sulfate	transurocanate	1-palmitoylglycerophosphoethanolamine
glutamine	indolepyruvate	1-stearoylglycerophosphoinositol
adrenate	3-phosphoglycerate	1-palmitoleoylglycerophosphocholine
myo-inositol	phenylacetylglycine	1-palmitoylplasmenylethanolamine
cysteine	phosphoenolpyruvate	Peptide TDTEDKGEFLSEGGGVR
ribose	12-HETE	4-methylphenylsulfate
4-methylphenyl	Indolyl-3-acryloylglycine	4-ethylphenyl

[0081] The ASD-related metabolites are involved in various metabolic pathways. Examples of metabolic pathways that the ASD-related metabolite can be involved in include, but are not limited to, amino acid metabolism, protein metabolism, carbohydrate metabolism, lipid metabolism, and metabolism of cofactors and vitamins. For example, the ASD-related metabolite can be a metabolite involved in glycine, serine and threonine metabolism; alanine and aspartate metabolism; glutamate metabolism; histidine metabolism; phenylalanine and tyrosine metabolism; tryptophan metabolism; valine, leucine and isoleucine metabolism; cysteine, methionine, SAM, and taurine metabolism; urea cycle; arginine-, proline-metabolism; and/or polyamine metabolism. The ASD-related metabolite can also be a

dipeptide or fibrinogen cleavage peptide. In addition, the ASD-related metabolite can be a metabolite involved in glycolysis, gluconeogenesis, pyruvate metabolism; and/or nucleotide sugars, pentose metabolism. The ASD-related metabolite can also be a metabolite involved in essential fatty acid, long chain fatty acid, monohydroxy and/or dicarboxylate fatty acid, eicosanoid, inositol, and/or lysolipid metabolism. The ASD-related metabolite can be a metabolite involved in hemoglobin and porphyrin metabolism, pantothenate and CoA metabolism, and/or benzoate metabolism.

[0082] In some embodiments, an ASD-related metabolite is a metabolite involved in tryptophan metabolism, a metabolite involved in fatty acid metabolism, or a metabolite involved in purine metabolism. In some embodiments, an ASD-related metabolite is glycolate, imidazole propionate, or N-acetylserine. In some embodiments, an ASD-related metabolite is 4-ethylphenylsulfate (4EPS), 4-ethylphenyl, indolepyruvate, indolyl-3-acryloylglycine, or serotonin. In some embodiments, an ASD-related metabolite is 4-methylphenylsulfate or 4-methylphenyl.

[0083] In some embodiments, the level of one ASD-related metabolite is adjusted or improving behavioral performance in the subject. For example, the level of 4EPS or indolepyruvate in the subject, for example the blood level (e.g., serum level) of 4EPS and indolepyruvate, can be adjusted for improving behavioral performance of the subject. In some embodiments, the level of two or more ASD-related metabolites is adjusted for improving behavioral performance in the subject. For example, the level of 4EPS and indolepyruvate in the subject, for example the blood level (e.g., serum level) of 4EPS and indolepyruvate, can be adjusted for improving behavioral performance of the subject.

[0084] Various methods can be used to adjust the level, for example blood level (e.g., serum level) or urine level, of the ASD-related metabolite in the subject for improving behavioral performance of the subject. For example, an antibody that specifically binds the ASD-related metabolite, an intermediate for the *in vivo* synthesis of the ASD-related metabolite, or a substrate for the *in vivo* synthesis of the ASD-related metabolite can be administered to the subject to adjust the level of the ASD-related metabolite in the subject. For example, an antibody that specifically binds 4EPS and/or one or more of the substrates and intermediates in the *in vivo* 4EPS synthesis can be used to reduce the level of 4EPS in the subject. In some embodiments, an antibody that specifically binds tyrosine,

hydroxyphenylpyruvic acid, *p*-coumaric acid, *p*-vinylphenynol, hydroxyphenylpropionate, and/or 4-ethylphenol is administered to the subject to reduce he level of 4EPS in the subject. In some embodiments, an antibody that specifically binds 4EPS is administered to the subject to reduce the level of 4EPS in the subject. As another example, an antibody that specifically binds 4-methylphenylsulfate and/or one or more of the substrates and intermediates in the *in vivo* 4-methylphenylsulfate synthesis can be used to reduce the level of 4-methylphenylsulfate in the subject. In some embodiments, an antibody that specifically binds tyrosine, hydroxyphenylpyruvic acid, hydroxyphenylpropionate, hydroxyphenylacetate, and/or *p*-cresol is administered to the subject to reduce the level of 4-methylphenylsulfate, e.g., the urine level of 4-methylphenylsulfate, in the subject. In some embodiments, an antibody that specifically binds 4-methylphenylsulfate is administered to the subject to reduce the level of 4-methylphenylsulfate in the subject. As yet another example, an antibody that specifically binds indolyl-3-acryloylglycine and/or one or more of the substrates and intermediates in the *in vivo* indolyl-3-acryloylglycine synthesis can be used to reduce the level of indolyl-3-acryloylglycine in the subject. In some embodiments, an antibody that specifically binds tryptophan, indolepyruvate, and/or indoleacrylic acid is administered to the subject to reduce the level of indolyl-3-acryloylglycine in the subject. In some embodiments, an antibody that specifically binds indolyl-3-acryloylglycine is administered to the subject to reduce the level of indolyl-3-acryloylglycine in the subject. As still yet another example, an antibody that specifically binds tryptophan and indolepyruvate can be used to reduce the level of indolepyruvate in the subject.

[0085] Methods for generating antibodies that specifically bind small molecules have been developed in the art. For example, generation of monoclonal antibodies against small molecules has been described in Rufo et al., J. Ag. Food Chem. 52:182-187 (2004), which is hereby incorporated by reference. For example, an animal such as a guinea pig or rat, preferably a mouse, can be immunized with a small molecule conjugated to a hapten (e.g., KLH), the antibody-producing cells, preferably splenic lymphocytes, can be collected and fused to a stable, immortalized cell line, preferably a myeloma cell line, to produce hybridoma cells which are then isolated and cloned. See, e.g., U.S. Patent No. 6,156,882, which is hereby incorporated by reference. In addition, the genes encoding the heavy and light chains of a small molecule-specific antibody can be cloned from a cell, e.g., the genes

encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody.

[0086] The level, for example blood level (e.g., serum level) or urine level, of the ASD-related metabolite in the subject can also be adjusted by inhibiting an enzyme involved in the *in vivo* synthesis of the ASD-related metabolite for improving behavioral performance of the subject.

[0087] As described herein, adjusting the level, for example blood level (e.g., serum level), of the ASD-related metabolite in the subject can ameliorate gastrointestinal (GI) distress of the subject suffering from ASD. The GI distress can be abdominal cramps, chronic diarrhea, constipation, intestinal permeability, or a combination thereof. As disclosed herein, amelioration is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, *e.g.*, symptom, associated with the pathological condition being treated. In some embodiments, the method can completely inhibited, *e.g.,* prevented from happening, or stopped, *e.g.,* terminated, such that the host no longer suffers from the pathological condition, or at least one or more of the symptoms that characterize the pathological condition. In some embodiments, the method can delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable.

[0088] As discussed above, gastrointestinal (GI) distress is an important comorbidity in ASD, given its prevalence and correlation with the severity of core autism behaviors. Also disclosed herein are methods for relieving gastrointestinal (GI) distress of a subject suffering from ASD. The methods can include reducing intestinal permeability in the subject. In some embodiments, the GI distress comprises abdominal cramps, chronic diarrhea, constipation, intestinal permeability, or a combination thereof. Reducing intestinal permeability can be achieved by altering the composition of gut microbiota in the subject. In some embodiments, altering the composition of gut microbiota in the subject comprises administering the subject a composition comprising bacteria, such as *Bacteroides* bacteria. In some embodiments, altering the composition of gut microbiota in the subject comprises fecal transplantation. In some embodiments, altering the composition of gut microbiota in the subject comprises probiotic treatment.

[0089] A variety of subjects are treatable. Generally, such subjects are mammals, where the term is used broadly to describe organisms which are within the class mammalia, including the orders carnivore (for example, dogs and cats), rodentia (for example, mice, guinea pigs and rats), and primates (for example, humans, chimpanzees and monkeys). In preferred embodiments, the subjects are humans.

[0090] In the methods disclosed herein, the amount of bacteria, for example *Bacteroides* bacteria (e.g., *B. fragilis*), administered to the subject in need of treatment can be determined according to various parameters such as the age, body weight, response of the subject, condition of the subject to be treated; the type and severity of the anxiety, ASD, or the pathological conditions with one or more symptoms of ASD; the form of the composition in which the bacteria is included; the route of administration; and the required regimen. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. A program comparable to that discussed above may be used in veterinary medicine. For example, the amount of bacteria can be titrated to determine the effective amount for administering to the subject in need of treatment. One of ordinary skill in the art would appreciate that the attending physician would know how to and when to terminate, interrupt or adjust administration of bacteria due to toxicity or organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity).

Methods for assessing the susceptibility of an ASD subject to probiotic treatment

[0091] Methods for assessing the susceptibility of a subject suffering from ASD to probiotic treatment are provided herein. The methods can include: determining the level of a *B. fragilis*-responsive metabolite in the subject; and comparing the level of the *B. fragilis*-responsive metabolite in the subject to a reference level of the metabolite in subjects suffering from ASD and one or more gastrointestinal disorders, wherein substantial identity between the blood level of the metabolites in the subject and the reference level indicates that the subject is susceptible to the probiotic treatment, for example *B. fragilis* probiotic treatment. In some embodiments, the method includes determining the level of two or more *B. fragilis*-responsive metabolites in the subject; and comparing the level of each of the two or more *B. fragilis*-responsive metabolites in the subject to the reference level of each of the two or more *B. fragilis*-responsive metabolites, wherein substantial identity between the

blood levels of the metabolites in the subject and the reference levels indicates an increased susceptibility of the subject to the probiotic treatment.

[0092] The level of the metabolite can be the level of the metabolite in circulation of the subject. For example, the level of the metabolite is the level of the metabolite in blood or other body fluids (e.g., cerebrospinal fluid, pleural fluid, amniotic fluid, semen, or saliva) of the subject. In some embodiments, the level of the metabolite is the blood level of the metabolite in the subject. The blood level of the metabolite can be, for example, serum level or plasma level of the metabolite. In some embodiments, the level of the metabolite is the urine level of the metabolite in the subject.

B. fragilis-responsive metabolites

[0093] As used herein, the term "*B. fragilis*-responsive metabolite" refers to a metabolite whose level has been determined to be altered by *B. fragilis* treatment. For example, the level of the metabolite may be altered in circulation of the subject after *B. fragilis* treatment. In some embodiments, the level of the metabolite is altered in blood, serum, plasma, body fluids (e.g., cerebrospinal fluid, pleural fluid, amniotic fluid, semen, or saliva), urine, and/or feces of the subject after *B. fragilis* treatment. The *B. fragilis*-responsive metabolite can be increased or decreased in level after *B. fragilis* treatment. In some instances, the ASD-related metabolite plays a causative role in the improvement of behavioral performance in the ASD subject treated with *B. fragilis*. In some instances, a *B. fragilis*-responsive metabolite is also an ASD-related metabolite. In some instances, an ASD-related metabolite is also a *B. fragilis*-responsive metabolite.

[0094] As disclosed herein, *B. fragilis*-responsive metabolite can be determined by comparing the pre-treatment level of a metabolite in a subject, for example a subject suffering from ASD, with the level of a metabolite in the subject after *B. fragilis* treatment. One of ordinary skill in the art will appreciate that variability in the level of metabolites may exist between individuals, and a reference level for a *B. fragilis*-responsive metabolite can be established as a value representative of the level of the metabolites in a population for ASD subjects suffering from one or more GI disorders for the comparison. In some embodiments, the *B. fragilis*-responsive metabolite has an increased level in the subject suffering from ASD as compared to the reference level. In some embodiments, the *B. fragilis*-responsive

metabolite has a decreased level in the subject suffering from ASD as compared to the reference level.

[0095] Non-limiting examples of *B. fragilis*-responsive metabolites are provided in Table 2.

Table 2. Exemplary *B. fragilis*-responsive metabolites

sarcosine (N-Methylglycine)	inosine
aspartate	adenosine
3-ureidopropionate	adenosine 5'-monophosphate (AMP)
glutarate (pentanedioate)	guanosine 5'- monophosphate (5'-GMP)
tyrosine	urate
3-(4-hydroxyphenyl)lactate	2'-deoxycytidine
3-phenylpropionate (hydrocinnamate)	uracil
serotonin (5HT)	pseudouridine
3-methyl-2-oxobutyrate	nicotinamide
3-methyl-2-oxovalerate	catechol sulfate
4-methyl-2-oxopentanoate	salicylate
isobutyrylcarnitine	equol sulfate
2-methylbutyroylcarnitine	erythritol
isovalerylcarnitine	dodecanedioate
2-hydroxybutyrate (AHB)	tetradecanedioate
arginine	hexadecanedioate
ornithine	octadecanedioate
2-aminobutyrate	undecanedioate
4-guanidinobutanoate	12-HETE
5-oxoproline	propionylcarnitine
glycylvaline	butyrylcarnitine
gamma-glutamyltryptophan	valerylcarnitine
TDTEDKGEFLSEGGGV	3-dehydrocarnitine
TDTEDKGEFLSEGGGVR	hexanoylcarnitine
sorbitol	octanoylcarnitine
pyruvate	choline
ribitol	chiro-inositol
ribose	pinitol
ribulose	3-hydroxybutyrate (BHBA)

xylitol	1,2-propanediol
citrate	1-linoleoylglycerophosphoethanolamine
fumarate	1-arachidonoylglycerophosphoethanolamine
malate	2-arachidonoylglycerophosphoethanolamine
linoleate (18:2n6)	1-stearoylglycerophosphoinositol
linolenate [alpha or gamma; (18:3n3 or 6)]	1-linoleoylglycerophosphoinositol
dihomo-linolenate (20:3n3 or n6)	1-arachidonoylglycerophosphoinositol
docosapentaenoate (n3 DPA; 22:5n3)	1-palmitoylplasmenylethanolamine
docosapentaenoate (n6 DPA; 22:5n6)	hypoxanthine
docosahexaenoate (DHA; 22:6n3)	eicosenoate (20:1n9 or 11)
heptanoate (7:0)	dihomo-linoleate (20:2n6)
pelargonate (9:0)	mead acid (20:3n9)
laurate (12:0)	adrenate (22:4n6)
myristate (14:0)	8-hydroxyoctanoate
palmitate (16:0)	3-hydroxydecanoate
palmitoleate (16:1n7)	16-hydroxypalmitate
margarate (17:0)	13-HODE + 9-HODE
stearate (18:0)	12,13-hydroxyoctadec-9(Z)-enoate
oleate (18:1n9)	9,10-hydroxyoctadec-12(Z)-enoic acid
stearidonate (18:4n3)	adipate
suberate (octanedioate)	2-hydroxyglutarate
sebacate (decanedioate)	pimelate (heptanedioate)
azelate (nonanedioate)	

Diagnosis of ASD

[0096] Also disclosed herein are methods for diagnosing ASD in a subject. In some embodiments, the methods include: determining the level of a cytokine in gut and the level of one or more ASD-related metabolites in the subject; and detecting whether or not there is an alteration in the level of the cytokine in gut and the level of at least one or more of the ASD-related metabolites in the subject as compared to a reference level of the cytokine and the metabolite in non-autistic subjects, whereby an alteration in the amount of the cytokine in gut and the level of at least one of the one or more metabolites indicates that the subject suffers from ASD.

[0097] In some embodiments, the method include: determining the level of an ASD-related metabolite in the subject; and detecting whether or not there is an alteration in the level of the ASD-related metabolite in the subject as compared to a reference level of the metabolite in non-autistic subjects, whereby an alteration in the level of the ASD-related metabolite indicates that the subject suffers from ASD. In some embodiments, the method include: determining the level of two or more ASD-related metabolites in the subject; and detecting whether or not there is an alteration in the level of the two or more ASD-related metabolites in the subject as compared to a reference level of the metabolites in non-autistic subjects, whereby an alteration in the level of at least two of the two or more ASD-related metabolites indicates that the subject suffers from ASD.

[0098] As disclosed herein, the level of the ASD-metabolite can be the level of the metabolite in circulation of the subject. For example, the level of the metabolite can be the level of the metabolite in blood or other body fluids (e.g., cerebrospinal fluid, pleural fluid, amniotic fluid, semen, or saliva) of the subject. In some embodiments, the level of the metabolite is the blood level of the metabolite in the subject. The blood level of the metabolite can be, for example, serum level or plasma level of the metabolite. In some embodiments, the level of the metabolite is the urine level of the metabolite in the subject.

[0099] One of ordinary skill in the art will appreciate that variability in the level of metabolites and/or the level of cytokines may exist between individuals in a non-autistic population. And thus, a reference level for the metabolite can be established as a value representative of the level of the metabolites in a non-autistic population for the comparison, and a reference level for the cytokine can be established as a value representative of the level of the cytokine in a non-autistic population for the comparison. In some embodiments, the ASD-related metabolite has an increased level in the subject suffering from ASD as compared to the reference level of the ASD-related metabolite. In some embodiments, the ASD-related metabolite has a decreased level in the subject suffering from ASD as compared to the reference level of the ASD-related metabolite. In some embodiments, the level of the cytokine is increased in the subject suffering from ASD as compared to the reference level of the cytokine. In some embodiments, the level of the cytokine is decreased in the subject suffering from ASD as compared to the reference level of the cytokine. The ASD-related

metabolites are described herein, and non-limiting examples of the ASD-related metabolites that can be used in the methods are provided in Table 1.

[0100] In some embodiments, the cytokine is interleukin-6 (IL-6). In some embodiments, the one or more ASD-related metabolites comprises a metabolite involved in tryptophan metabolism, a metabolite involved in fatty acid metabolism, a metabolite involved in purine metabolism, glycolate, imidazole propionate, N-acetylserine, or any combination thereof. Non-limiting examples of metabolites involved in tryptophan metabolism include 4-ethylphenylsulfate, indolepyruvate, indolyl-3-acryloylglycine, or serotonin. In some embodiments, the ASD-related metabolite is 4-ethylphenylsulfate, indolepyruvate, indolyl-3-acryloylglycine, or serotonin.

[0101] In the methods disclosed in the present disclosure, the level of a metabolite in the subject can be determined by any conventional methods known in the art, including but not limited to chromatography, liquid chromatography, size exclusion chromatography, high performance liquid chromatography (HPLC), gas chromatography, mass spectrometry, tandem mass spectrometry, matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, electrospray ionization (ESI) mass spectrometry, surface-enhanced laser deorption/ionization-time of flight (SELDI-TOF) mass spectrometry, quadrupole-time of flight (Q-TOF) mass spectrometry, atmospheric pressure photoionization mass spectrometry (APPI-MS), Fourier transform mass spectrometry (FTMS), matrix-assisted laser desorption/ionization-Fourier transform-ion cyclotron resonance (MALDI-FT-ICR) mass spectrometry, secondary ion mass spectrometry (SIMS), radioimmunoassays, microfluidic chip-based assay, detection of fluorescence, detection of chemiluminescence, or a combination thereof.

EXAMPLES

[0102] Some aspects of the embodiments discussed above are disclosed in further detail in the following examples, which are not in any way intended to limit the scope of the present disclosure.

Experimental Material and Methods

[0103] The following experimental methods were used for Examples 1-8 described below.

Animals and MIA

[0104] Pregnant C57BL/6N (Charles River; Wilmington, MA) were selected at random from a larger cohort of pregnant females, and injected i.p. on E12.5 with saline or 20 mg/kg poly(I:C) according to the methods described in Smith et al., 2007. All animal experiments were approved by the Caltech IACUC.

B. fragilis treatment

[0105] At 3 weeks of age, saline and poly(I:C) offspring across individual litters were weaned into cages of 4 non-littermate offspring of the same treatment group to generate a randomized experimental design (Lazic, 2013). Cages within the poly(I:C) versus saline treatment groups were selected at random for treatment with *B. fragilis* or vehicle, every other day for 6 days. To preclude any confounding effects of early life stress on neurodevelopment and behavior, suspensions were not administered by oral gavage. For *B. fragilis* treatment, 10^10 cfu freshly grown *B. fragilis* was suspended in 1 mL 1.5% sodium bicarbonate, mixed with 4 ml sugar-free applesauce and spread over four standard food pellets. For vehicle treatment, saline and poly(I:C) animals were fed 1.5% sodium bicarbonate in applesauce over food pellets. Applesauce and pellets were completely consumed by mice of each treatment group by 48 hours after administration. The same procedure was used for treatment with mutant *B. fragilis* lacking PSA and *B. thetaiotaomicron.*

Intestinal permeability assay

[0106] Adult mice were fasted for 4 hours before oral gavage with 0.6 g/kg 4 kDa FITC-dextran (Sigma Aldrich). 4 hours later, blood samples were collected by cardiac puncture and spun through SST vacutainers (Becton Dickinson). FITC-dextran standards and 3X-diluted sera were immediately read for FITC fluorescence intensity at 521 nm using an xFluor4 spectrometer (Tecan). Mice were fed 3% dextran sulfate sodium salt (DSS; MP Biomedicals) in drinking water for 7 days to chemically induce colitis.

In vitro immune assays

[0107] Methods for Treg and Gr-1 flow cytometry and CD4+ T cell *in vitro* stimulation are described in Hsiao et al., 2012. Briefly, cells were harvested in complete RPMI from spleens and mesenteric lymph nodes. For subtyping of splenocytes, cells were stained with Gr-1 APC, CD11b-PE, CD4-FITC and Ter119-PerCP-Cy5.5 (Biolegend). For detection of Tregs, splenocytes were stimulated for 4 hours with PMA/ionomycin in the

presence of GolgiPLUG (BD Biosciences), blocked for Fc receptors and labeled with CD4-FITC, CD25-PE, Foxp3-APC and Ter119-PerCP-Cy5.5. Samples were processed using the FACSCalibur cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). For CD4+ T cell stimulation assays, 10^6 CD4+ T cells were cultured in complete RPMI with PMA (50 ng/ml) and ionomycin (750 ng/ml) for 3 days at 37 °C with 5% (vol/vol) CO_2. Each day, supernatant was collected for ELISA assays to detect IL-6 and IL-17, according to the manufacturer's instructions (eBioscience).

IL-6 oral gavage and in vitro colon culture

[0108] For *in vivo* assays, adult mice were gavaged with 5 ug carrier-free recombinant mouse IL-6 (eBioscience) in 1.5% sodium bicarbonate. At 4 hours post-gavage, 1 cm regions of distal, medial and proximal colon were dissected, washed in HBSS and processed for qRT-PCR, as described above. For in vitro assays, adult mice were sacrificed and 1 cm regions of distal, medial and proximal colon were dissected, washed and bisected for colon culture with 0-80 ng/ml IL-6 in complete RPMI media. After 4 hours of culture, colon pieces were harvested and processed for qRT-PCR, as described above.

Intestinal qRT-PCR, Western blots, and cytokine profiles

[0109] 1 cm regions of the distal, medial and proximal colon and small intestine were washed in HBSS and either a) homogenized in ice-cold Trizol for RNA isolation and reverse transcription according to Hsiao and Patterson, 2011, or b) homogenized in Tissue Extraction Reagent I (Invitrogen) containing EDTA-free protease inhibitors (Roche) for protein assays. For SYBR green qRT-PCR, validated primer sets were obtained from Primerbank (Harvard). For cytokine profiling, mouse 20-plex cytokine arrays (Invitrogen) were run on the Luminex FLEXMAP 3D platform by the Clinical Immunobiology Correlative Studies Laboratory at the City of Hope (Duarte, CA). Western blots were conducted according to standard methods and probed with rabbit anti-claudin 8 or rabbit anti-claudin 15 (Invitrogen) at 1:100 dilution.

Microbial DNA extraction, 16S rRNA gene amplification and pyrosequencing

[0110] Bacterial genomic DNA was extracted from mouse fecal pellets using the MoBio PowerSoil Kit following protocols benchmarked as part of the NIH Human Microbiome Project. The V3-V5 regions of the 16S rRNA gene were PCR amplified using individually barcoded universal primers containing linker sequences for 454-pyrosequencing.

Sequencing was performed at the Human Genome Sequencing Center at Baylor College of Medicine using a multiplexed 454-Titanium pyrosequencer.

16S rRNA Gene Sequence Analysis

[0111] FASTA and quality files were obtained from the Alkek Center for Metagenomics and Microbiome Research at the Baylor College of Medicine and quality filtered. Sequences <200bp and >1000bp, and sequences containing any primer mismatches, barcode mismatches, ambiguous bases, homopolymer runs exceeding six bases, or an average quality score of below 30 were discarded. Quality filtered sequences were then analyzed using the QIIME 1.6 software package (Caporaso et al., 2010b). Sequences were then checked for chimeras and clustered to operational taxonomic units (OTUs) using the USearch pipeline (Edgar, 2010; Edgar et al., 2011) with a sequence similarity index of 97%. OTUs were subsequently assigned taxonomic classification using the basic local alignment search tool (BLAST) classifier (Altschul et al, 1990), based on the small subunit non-redundant reference database release 111 (Quest et al, 2013) with 0.001 maximum e-value. These taxonomies were then used to generate taxonomic summaries of all OTUs at different taxonomic levels. For tree-based alpha- and beta diversity analyses, representative sequences for each OTU were aligned using PyNAST (Caporaso et al., 2010a) and a phylogenetic tree was constructed based on this alignment using FastTree (Price et al., 2009). Alpha diversity estimates (by Observed Species and Faith's phylogenetic diversity [PD]; (Faith, 1992)) and evenness (by Simpson's evenness and Gini Coefficient; (Wittebolle et al., 2009)) were calculated and compared between groups using a nonparametric test based on 100 iterations using a rarefaction of 2082 sequences from each sample. For beta diversity, even sampling of 2160 sequences per sample was used, and calculated using weighted and unweighted UniFrac (Lozupone and Knight, 2005). Beta Diversity was compared in a pairwise fashion (Saline (S) vs Poly(I:C) (P), Poly(I:C) (P) vs Poly(I:C) + *B. fragilis* treatment (P+BF)) using the Analysis of Similarity (ANOSIM; Fierer et al 2010) with 999 permutations to determine statistical significance.

Identification of differences in specific OTUs

[0112] Key OTUs, that discriminate between Saline and Poly(I:C) treatment groups, and between Poly(I:C) and Poly(I:C) + *B. fragilis* treatment groups, were identified using an unbiased method from OTU tables, generated by QIIME, using three

complimentary analyses: (1) Metastats comparison (White et al., 2009), (2) the Random Forests algorithm, first under QIIME (Knights et al., 2011) and subsequently coupled with Boruta feature selection, in the Genboree microbiome toolset (Riehle et al., 2012), and (3) the Galaxy platform-based LDA Effect Size analysis (LEfSe;(Segata et al., 2011)). Only OTUs that differ significantly between treatment groups were candidates for further analyses ($p<0.05$ for (1) and (3), and >0.0001 mean decrease in accuracy for Random Forests and subsequent identification by the Boruta algorithm). Metastats analyses were done using the online interface (http://metastats.cbcb.umd.edu) with QIIME-generated OTU tables of any two treatment groups. The Random Forests algorithm was used to identify discriminatory OTUs in the QIIME software package (Breiman, 2001; Knights et al., 2011), comparing two treatment groups at a time, based on 1000 trees and a 10-fold cross-validation, and was further validated and coupled with the Boruta feature selection algorithm, as implemented in the Genboree Microbiome toolset (Kursa and Rudnicki, 2010; Riehle et al., 2012). Only those OTUs that were confirmed by the Boruta algorithm were defined as discriminatory. The ratio between observed and calculated error rates was used as a measure of confidence for Random Forests Analyses: this ratio was 5.0 for saline vs. poly(I:C) (with an estimated error of 0.1±0.21) and 2.86 for poly(I:C) vs. poly(I:C) + *B. fragilis* (with an estimated error of 0.23±0.22). In order to overcome any mislabeling by any one of the three methods only OTUs that were identified by at least two of the three above methods were defined as discriminatory. For the analyses in Figure 1, OTUs that were significantly altered by MIA were identified by comparing the saline vs. poly(I:C) groups. For the analyses in Figure 6, the poly(I:C) vs. poly(I:C)+*B. fragilis* groups were compared, and only report only those OTUs that have also been identified by the analyses in Figure 1.

[0113] Key OTUs were than aligned using the SINA aligner (http://www.arb-silva.de/aligner/; Pruesse et al., 2012), compared to the SILVA reference database release 111 (Quast et al., 2013) using Arb (Ludwig et al., 2004) and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Heat maps of key OTUs were generated by extracting their relative abundance from the OTU table. These data were then normalized (so that the sum of squares of all values in a row or column equals one), first by OTU and subsequently by sample, and clustered by correlation using Cluster 3.0 (de Hoon et al., 2004). Finally, abundance data was visualized using Java TreeView (Saldanha, 2004).

B. fragilis colonization assay

[0114] Fecal samples were sterilely collected from MIA and control offspring at 1, 2 and 3 weeks after the start of treatment with *B. fragilis* or vehicle. Germ-free mice were treated with *B. fragilis* as described above to serve as positive controls. DNA was isolated fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen). 50 ng DNA was used for qPCR with *B. fragilis*-specific, 5' TGATTCCGCATGGTTTCATT 3' (SEQ ID NO: 1) and 5' CGACCCATAGAGCCTTCATC 3' (SEQ ID NO: 2), and universal 16S primers 5' ACTCCTACGGGAGGCAGCAGT 3' (SEQ ID NO: 3) and 5' ATTACCGCGGCTGCTGGC 3' (SEQ ID NO: 4) according to Odamaki et al., 2008.

Behavioral testing

[0115] Adult MIA and control offspring were behaviorally tested as described in Hsiao et al., 2012 and Malkova et al., 2012. Mice were tested beginning at 6 weeks of age for pre-pulse inhibition, open field exploration, marble burying, social interaction and adult ultrasonic vocalizations, in that order, with at least 5 days between behavioral tests. Behavioral data for *B. fragilis* treatment and control groups (Figure 10) represent cumulative results collected from multiple litters of 3-5 independent cohorts of mice for PPI and open field tests, 2-4 cohorts for marble burying, 2 cohorts for adult male ultrasonic vocalization and 1 cohort for social interaction. Discrepancies in sample size across behavioral tests reflect differences in when during our experimental study a particular test was implemented.

[0116] *Pre-pulse inhibition.* PPI tests are used as a measure of sensorimotor gating and were conducted and analyzed according to the procedure described in Geyer and Swerdlow, 2001 and Smith et al., 2007. Briefly, mice were acclimated to the testing chambers of the SR-LAB startle response system (San Diego Instruments) for 5 minutes, presented with six 120 db pulses of white noise (startle stimulus) and then subjected to 14 randomized blocks of either no startle, startle stimulus only, 5 db prepulse with startle or 15 db prepulse with startle. The startle response was recorded by a pliezo-electric sensor, and the percent PPI is defined as: [((startle stimulus only – 5 or 15 db prepulse with startle)/startle stimulus only)*100].

[0117] *Open field exploration.* The open field test is widely used to measure anxiety-like and locomotor behavior in rodents. Mice were placed in 50 x 50 cm white Plexiglas boxes for 10 minutes. An overhead video camera recorded the session, and

Ethovision software (Noldus) was used to analyze the distance traveled, and the number of entries and duration of time spent in the center arena (central 17 cm square).

[0118] *Marble burying.* Marble burying is an elicited repetitive behavior in rodents analogous to those observed in autistic individuals (Silverman et al., 2010b). This test was conducted and analyzed according to methods described in Thomas et al., 2009 and Malkova et al., 2012. Mice were habituated for 10 minutes to a novel testing cage containing a 4 cm layer of chipped cedar wood bedding and then transferred to a new housing cage. 18 glass marbles (15 mm diameter) were aligned equidistantly 6 x 3 in the testing cage. Mice were returned to the testing cage and the number of marbles buried in 10 minutes was recorded.

[0119] *Sociability and social preference.* Social interaction tests were conducted and analyzed according to methods adopted from Sankoorikal et al., 2006 and Yang et al., 2011. Briefly, testing mice were habituated for 10 minutes to a 40 x 60 cm Plexiglas three-chambered apparatus containing clear interaction cylinders in each of the side chambers. Sociability was tested in the following 10 minute session, where the testing mouse was given the opportunity to explore a novel same-sex, age-matched mouse in one interaction cylinder (social object) versus a novel toy (green sticky ball) in the other interaction cylinder of the opposite chamber. Social preference was tested in the final 10 minute session, where the testing mouse was given the opportunity to explore a now familiar mouse (stimulus mouse from the previous sociability session) versus a novel unfamiliar same-sex, age-matched mouse. In each session, the trajectory of the testing mouse was tracked with Ethovision software (Noldus). Sociability data is presented as preference for the mouse over the toy: percent of time in the social chamber - percent of time in the nonsocial chamber, and social preference data is presented as preference for the unfamiliar mouse over the familiar mouse: percent of time in the unfamiliar mouse chamber – percent of time in the familiar mouse chamber. Similar indexes were measured for chamber entries, and entries into and duration spent in the contact zone (7 x 7 cm square surrounding the interaction cylinder).

[0120] *Adult ultrasonic vocalizations.* Male mice produce USVs in response to female mice as an important form of communication (Portfors, 2007). Adult male USV production in response to novel female exposure was measured according to methods described in Grimsley et al., 2011; Scattoni et al., 2011; and Silverman et al., 2010a. Adult

males were single-housed one week before testing and exposed for 20 minutes to an unfamiliar adult female mouse each day starting four days prior to testing in order to provide a standardized history of sexual experience and to adjust for differences in social dominance. On testing day, mice were habituated to a novel cage for 10 minutes before exposure to a novel age-matched female. USVs were recorded for 3 minutes using the UltraSoundGate microphone and audio system (Avisoft Bioacoustics). Recordings were analyzed using Avisoft's SASLab Pro software after fast Fourier transformation at 512 FFT-length and detection by a threshold-based algorithm with 5 ms hold time. Data presented reflect duration and number of calls produced in the 3 minute session.

Metabolomics screening

[0121] Sera were collected by cardiac puncture from behaviorally validated adult mice. Samples were extracted and analyzed on GC/MS, LC/MS and LC/MS/MS platforms by Metabolon, Inc. Protein fractions were removed by serial extractions with organic aqueous solvents, concentrated using a TurboVap system (Zymark) and vacuum dried. For LC/MS and LC/MS/MS, samples were reconstituted in acidic or basic LC-compatible solvents containing >11 injection standards and run on a Waters ACQUITY UPLC and Thermo-Finnigan LTQ mass spectrometer, with a linear ion-trap front-end and a Fourier transform ion cyclotron resonance mass spectrometer back-end. For GC/MS, samples were derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Chemical entities were identified by comparison to metabolomic library entries of purified standards. Following log transformation and imputation with minimum observed values for each compound, data were analyzed using two-way ANOVA with contrasts.

4EPS synthesis and detection

[0122] Potassium 4-ethylphenylsulfate was prepared using a modification of a procedure reported for the synthesis of aryl sulfates in Burlingham et al., 2003 and Grimes, 1959 (Figure 15A). 4-ethylphenol (Sigma-Aldrich, 5.00 g, 40.9 mmol) was treated with sulfur trioxide-pyridine complex (Sigma-Aldrich, 5.92 g, 37.2 mmol) in refluxing benzene (20 ml, dried by passing through an activated alumina column). After 3.5 hours, the resulting solution was cooled to room temperature, at which point the product crystallized. Isolation

by filtration afforded 7.93 g of crude pyridinium 4-ethylphenylsulfate as a white crystalline solid. 1.00 g of this material was dissolved in 10 mL of 3% triethylamine in acetonitrile and filtered through a plug of silica gel (Silicycle, partical size 32-63 μm), eluting with 3% triethylamine in acetonitrile. The filtrate was then concentrated, and the resulting residue was dissolved in 20 mL of deionized water and eluted through a column of Dowex 50WX8 ion exchange resin (K^+ form), rinsing with 20 mL of deionized water. The ion exchange process was repeated once more, and the resulting solution concentrated under vacuum to afford 618 mg (55% overall yield) of potassium 4-ethylphenylsulfate as a white powder (Figure 15A).

[0123] ^1H and ^{13}C NMR spectra of authentic potassium 4-ethylphenylsulfate were recorded on a Varian Inova 500 spectrometer and are reported relative to internal DMSO-d_5 (^1H, δ = 2.50; ^{13}C, δ = 39.52). A high-resolution mass spectrum (HRMS) was acquired using an Agilent 6200 Series TOF with an Agilent G1978A Multimode source in mixed ionization mode (electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)). Spectroscopic data for potassium 4-ethylphenylsulfate are as follows: ^1H NMR (DMSO-d_6, 500 MHz) δ 7.11 – 7.04 (m, 4H), 2.54 (q, J = 7.6 Hz, 2H), 1.15 (t, J = 7.6 Hz, 3H); ^{13}C NMR (DMSO-d_6, 126 MHz) δ 151.4, 138.3, 127.9, 120.6, 27.5, 16.0; HRMS (Multimode-ESI/APCI) calculated for $C_8H_9O_4S$ $[M–K]^-$ 201.0227, found 201.0225.

[0124] Authentic 4EPS and serum samples were analyzed by LC/MS using an Agilent 110 Series HPLC system equipped with a photodiode array detector and interfaced to a model G1946C single quadrupole expectospray mass spectrometer. HPLC separations were obtained at 25°C using an Agilent Zorbax XDB-C18 column (4.6 mm x 50 mm x 5 um particle size). The 4EPS ion was detected using selected ion monitoring for ions of m/z 200.9 and dwell time of 580 ms/ion, with the electrospray capillary set at 3 kV. Authentic potassium 4EPS was found to possess a retention time of 6.2 minutes when eluted in 0.05% trifluoroacetic acid and acetonitrile, using a 10-minute linear gradient from 0-50% acetonitrile. For quantification of 4EPS in mouse sera, a dose-response curve was constructed by plotting the total ion count peak area for known concentrations of authentic potassium 4EPS against the analyte concentration (R^2=0.9998; Figure 15B). Mouse serum samples were diluted four-fold with acetonitrile and centrifuged at 10,000 g at 4°C for 3 minutes. 10 ul of supernatant was injected directly into the HPLC system.

4EPS sufficiency experiments

[0125] Wildtype mice were injected i.p. with saline or 30 mg/kg 4EPS potassium salt daily from 3 to 6 weeks of age. A dose-response curve was generated by measuring serum 4EPS levels at various times after i.p. injection of 30 mg/kg 4EPS (Figure 15C). Mice were behaviorally tested as described above from 6 to 9 weeks of age.

Statistical Analysis

[0126] Statistical analysis was performed using Prism software (Graphpad). Data were assessed for normal distribution and plotted in the figures as mean ± SEM. Differences between two treatment groups (i.e. control versus 4EPS) were assessed using two-tailed, unpaired Student t test with Welch's correction. Differences among multiple groups (saline versus poly(I:C) versus poly(I:C)+*B. fragilis*/*B. thetaiotaomicron*) were assessed using one-way ANOVA with Bonferroni post hoc test. Two-way repeated measures ANOVA with Bonferroni post hoc test was used for analysis of PPI and CD4+ T-cell stimulation data. Two-way ANOVA with contrasts was used for analysis of the metabolite data. Sample sizes denote the number of individual mice per treatment group, given the individual randomization design of the study (Lazic, 2013). Significant differences emerging from the above tests are indicated in the figures by *$p<0.05$, **$p<0.01$, ***$p<0.001$. Notable near-significant differences ($0.5<p<0.1$) are indicated in the figures. Notable non-significant (and non-near significant) differences are indicated in the figures by "n.s.".

Example 1

Offspring of immune-activated mothers exhibit GI symptoms of human ASD

[0127] Adult MIA offspring, which exhibit cardinal behavioral and neuropathological symptoms of ASD (Malkova et al., 2012), were also found to display a significant deficit in intestinal barrier integrity, as reflected by increased translocation of orally administered FITC-dextran across the intestinal epithelial layer and into the circulation (Figure 1A, left panel). This MIA-associated increase in intestinal permeability is similar to what's seen in mice treated with dextran sodium sulfate (DSS), a chemical used to induce experimental colitis (Figure 1A, left panel) (Wirtz et al., 2007). Deficits in intestinal integrity were detectable in 3-week-old MIA offspring (Figure 1A, right panel), indicating that the abnormality was established during early life. To assess the molecular basis for increased intestinal permeability in MIA offspring, colons of MIA offspring were examined for the tight junction components ZO-1 (TJP1), ZO-2 (TJP2), ZO-3 (TJP3), occludin and claudins

(CLDN) 1, 2, 3, 4, 7, 8, 12, 13 and 15 (Holmes et al., 2006). Consistent with the leaky gut phenotype found in subsets of ASD children displaying GI abnormalities, colons from adult MIA offspring exhibited decreased expression of transcripts for ZO-1, ZO-2, occludin and claudin 8, and increased expression of claudin 15 mRNA (Figure 1B). Deficient expression of ZO-1 is also observed in small intestines of adult MIA offspring (Figure 2A), demonstrating a widespread defect in intestinal barrier integrity.

[0128] Increased permeability is observed in several intestinal diseases, as well as subsets of ASD, and is commonly associated with signs of inflammation (Hering et al., 2012; Turner, 2009; White, 2003). In addition to changes in expression of tight junction components, colons from adult MIA offspring were found to display increased levels of interleukin-6 (IL-6) mRNA and protein (Figures 1C and 1D) and decreased levels of the cytokines/chemokines IL-12p40/p70, IP-10, MIG and MIP-1a (Figure 1D). Small intestines from MIA offspring also exhibit altered cytokine/chemokine profiles (Figure 2C). Changes in intestinal cytokines were not accompanied by overt GI pathology, as assessed by histological examination of gross epithelial morphology from hematoxylin- and eosin-stained sections. Consistent with the alterations in immune-related signaling factors, however, mesenteric lymph nodes and spleens from adult MIA offspring were found to contain decreased levels of regulatory T cells and hyper-responsive production of IL-6 and IL-17 by CD4+ T helper cells, suggestive of a pro-inflammatory phenotype (Figure 3A-D) (Hsiao et al., 2012). Similar findings supporting enteric immune activation are seen in subsets of ASD individuals (Onore et al., 2012).

[0129] In view of the foregoing, this examples shows that adult offspring of immune-activated mothers exhibit increased gut permeability and abnormal intestinal cytokine profiles, recapitulating ASD-related GI symptoms in a mouse model.

Example 2

MIA Offspring display dysbiosis of the gut microbiota

[0130] The potential link between disruption of the normal gut microbiota and GI dysfunction in an ASD mouse model was examined in this example.

[0131] To evaluate whether MIA induces microbiota alterations, the fecal bacterial population was surveyed by 16S rRNA gene sequencing of samples isolated from adult offspring of mothers treated with poly(I:C) or saline. Alpha diversity, i.e., species

richness and evenness, did not differ significantly between control and MIA offspring, as measured by Faith's phylogenetic diversity (PD) index, and number of Observed Species (p=1.0000 and 0.2790, respectively) and the Gini coefficient and Simpson evenness index (p=0.5430 and p=0.2610, respectively; Figures 4A and 4B). In contrast, unweighted UniFrac analysis, which measures the degree of phylogenetic similarity between microbial communities, reveals a strong effect of MIA on the gut microbiota of adult offspring (Figure 5A-E). MIA samples cluster distinctly from controls by principal coordinate analysis (PCoA; ANOSIM R=0.2829, p=0.0030), indicating robust changes in the membership of gut bacteria from MIA offspring compared to controls (Figure 5A). The effect of MIA on altering the composition of the gut microbiota is further evident when sequences from the classes Clostridia and Bacteroidia, which account for approximately 90.1% of the total reads in our survey (46,484 reads out of 51,586 in the S and P groups), were exclusively examined by PCoA (R=0.2331, p=0.0070; Figure 5B), but not when Clostridia and Bacteroidia sequences were specifically excluded from PCoA of all other bacterial classes (R=0.1051, p=0.0700; Figure 5C). This indicates that changes in the diversity of Clostridia and Bacteroidia operational taxonomic units (OTUs) are the primary drivers of gut microbiota differences between MIA offspring and controls.

[0132] 67 OTUs out of the 1474 OTUs detected across any of the samples discriminate between treatment groups, including those assigned to the bacterial families Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae, Alcaligenaceae, Porphyromonadaceae, Prevotellaceae, and Rikenellaceae, and unclassified Bacteroidales (Figure 5D). Of these 67 discriminatory OTUs, 19 are more abundant in control samples and 48 are more abundant in MIA samples. Consistent with the PCoA results (Figures 5A-C), the majority of OTUs that discriminate MIA offspring from controls are assigned to the classes Bacteroidia (45 of 67 OTUs; 67.2%) and Clostridia (17 of 67 OTUs; 25.4%), whereas the few remaining discriminatory OTUs belong to Proteobacteria (3 OTUs; 4.5%) and other classes (Tenericutes and unclassified, 1 OTU each; 3.0%). Interestingly, *Porphyromonadaceae*, *Prevotellaceae*, and many unclassified Bacteriodales (36 of the 45 discriminatory Bacteroidial OTUS; 80%), and *Lachnospiriceae* (8 of the 14 discriminatory Clostridial OTUs; 57%) were more abundant in MIA offspring. Conversely, *Ruminococcaceae* (2 OTUs), *Erysipelotrichaceae* (2 OTUs), and the beta Proteobacteria

family *Alcaligenaceae* (2 OTUs) were more abundant in control offspring (Figure 5D). These data indicate that specific Lachnospiraceae, along with other Bacteroidial species, play an important role in MIA pathogenesis, while other taxa may have a protective role. Importantly, there is no significant difference in the overall relative abundance of Clostridia (13.63 ± 2.54% vs 14.44 ± 2.84% mean ± SEM; Student's t-test p=0.8340) and Bacteroidia (76.25 ± 3.22% vs 76.22 ± 3.46% mean ± SEM; Student's t-test p=0.9943) between MIA offspring and controls (Figure 5E, left panel), indicating that alterations in the membership of rare OTUs drive major changes in the gut microbiota between experimental groups.

[0133] Differences in taxonomic diversity was also seen in less prominent bacterial classes, with MIA offspring displaying significantly decreased relative abundance of Erysipelotrichi (0.15 ± 0.03 % v.s. 0.74 ± 0.25 % mean ± SEM; Student's t-test p-value=0.0334) compared to controls (Figure 5E, right panel). Overall, MIA was found to lead to dysbiosis of the gut microbiota, driven primarily by alterations in specific OTUs of the bacterial classes Clostridia and Bacteroidia. Changes in OTUs classified as Lachnospiraceae and Ruminococcaceae of the order Clostridiales parallel reports of increased Clostridium species in the feces of subjects with ASD (Finegold et al., 2012). Altogether, modeling MIA as a primary autism risk factor in mice induces not only behavioral and neuropathological features of ASD (Boksa, 2010), but also GI symptoms analogous to those described in subsets of ASD individuals. The data presented herein shows that MIA can be used as a model for human ASD with comorbid GI issues.

Example 3

B. fragilis treatment improves gut barrier integrity in MIA offspring

[0134] Gut microbes play an important role in the development, maintenance and repair of the intestinal epithelium (Sharma et al., 2010; Turner, 2009). To determine whether targeting the gut microbiota could impact the development or persistence of MIA-associated GI abnormalities, offspring was treated with the human commensal bacterium *B. fragilis* at weaning, and then tested for GI abnormalities at 8 weeks of age. Remarkably, *B. fragilis* treatment corrected intestinal permeability in MIA offspring (Figure 6A). In addition, *B. fragilis* treatment ameliorated MIA-associated changes in gene expression of CLDNs 8 and 15, but had no significant effect on expression levels of TJP1, TJP2 or OCLN mRNA (Figure 6B). Similar changes are observed in protein levels of claudin 8 and 15 in the colon, with

restoration by *B. fragilis* treatment (Figures 6C-D). No such effects of *B. fragilis* on tight junction expression are observed in small intestines from MIA offspring (Figure 2B), consistent with the fact that *Bacteroides* species are predominantly found in the colon. Also, the presence of GI defects prior to probiotic administration (Figure 1A, right panel) suggests that *B. fragilis* can treat ASD-related pathology in MIA offspring.

[0135] *B. fragilis* treatment also restored MIA-associated increases in colon IL-6 mRNA and protein levels to those found in control mice (Figures 6E-F). Levels of other cytokines were altered in both colons and small intestines of MIA offspring (Figures 1D and 2C), but these were not affected by *B. fragilis* treatment, revealing specificity for IL-6. This finding is consistent with a critical role for IL-6 in the MIA model (Smith et al., 2007). Altered intestinal cytokine profiles may form the basis for the increased intestinal permeability observed in MIA offspring, as several cytokines including IL-6 are reported to modulate tight junctions and regulate intestinal barrier integrity (Suzuki et al., 2011; Turner, 2009). It was further found that recombinant IL-6 treatment can modulate colon levels of both claudin 8 and claudin 15 *in vivo* and in *in vitro* colon organ cultures (Figure 7A-C), suggesting that *B. fragilis*-mediated restoration of colonic IL-6 levels could underlie its effects on gut permeability. Collectively, these findings demonstrate that *B. fragilis* treatment of MIA offspring reverses defects in GI barrier integrity, and corrects alterations in tight junction and cytokine expression.

Example 4

B. fragilis treatment restores microbiota changes in MIA offspring

[0136] In addition to ameliorating GI physiology in MIA offspring, *B. fragilis* treatment induces long-term effects on the composition of the intestinal microbiota. No significant differences were observed at the global level by PCoA (ANOSIM R=0.0060 p=0.4470) or in microbiota richness (PD: p=0.2980, Observed Species: p=0.5440) and evenness (Gini: p=0.6110, Simpson Evenness: p=0.5600; Figures 8A, 4A-B). However, corrective effects of *B. fragilis* treatment were apparent upon evaluating specific key OTUs that discriminate adult MIA offspring from controls (Figure 8B). Specifically, MIA offspring treated with *B. fragilis* displayed complete restoration in the relative abundance of 6 out of the 67 OTUs discriminate MIA from control offspring (28 other OTUs, not identified as discriminatory between MIA and control offspring, could discriminate between MIA

offspring and those that have been treated with *B. fragilis*). These 6 OTUs are taxonomically assigned as unclassified Bacteroidia and Clostridia of the family Lachnospiraceae (Figure 8B). Notably, these alterations occurred in the absence of persistent colonization of *B. fragilis*, which remains undetectable in fecal and cecal samples isolated from treated MIA offspring, as assessed by quantitative real-time PCR (Figure 9A-B). Interestingly, 4 of the 10 Lachnospiraceae elevated in MIA offspring were corrected by *B. fragilis* treatment (Figures 5D and 8A-C). In addition, *B. fragilis* treatment restored the relative abundance of 2 Bacteroidia OTUs to levels observed in controls (Figure 8B). Phylogenetic reconstruction of the 6 OTUs that were altered by MIA and restored by *B. fragilis* treatment reveals that the two Bacteroidia OTUs cluster together into a monophyletic group (Figure 8D). In addition, the Lachnospiraceae OTUs that were significantly altered by MIA and corrected by *B. fragilis* cluster into 2 seperate monophyletic groups (Figure 8D). These results indicate that, although treatment of MIA offspring with *B. fragilis* may not lead to persistent colonization of *B. fragilis* itself, it can correct the relative abundance of specific groups of related microbes of the Lachnospiraceae family as well as unclassified Bacteriodales.

[0137] Altogether, this example demonstrates that treatment of MIA offspring with *B. fragilis* can ameliorate particular changes involved in MIA-associated dysbiosis of the commensal microbiota and correct GI abnormalities similar to those observed in subsets of autistic individuals.

Example 5

B. fragilis treatment corrects ASD-related behavioral abnormalities

[0138] To explore the potential impact of GI dysfunction on core ASD behavioral abnormalities, the question whether *B. fragilis* treatment impacts ASD-related behaviors in MIA offspring was investigated.

[0139] Adult MIA offspring were found to display cardinal behavioral features of ASD in a variety of behavioral assays. Open field exploration involves mapping an animal's movement in an open arena to measure of locomotion and anxiety (Bailey and Crawley, 2009). MIA offspring displayed decreased entries and time spent in the center of the arena, but no difference in the total distance traveled, which is indicative of anxiety-like behavior (Figure 10A; compare saline (S) to poly(I:C) (P)). The pre-pulse inhibition (PPI) task measures the ability of an animal to inhibit its startle in response to an acoustic tone

("pulse") when it is preceded by a lower-intensity stimulus ("pre-pulse"). Deficiencies in PPI are a measure of impaired sensorimotor gating, and are observed in several neurodevelopmental disorders, including autism (Perry et al., 2007). MIA offspring exhibited decreased PPI in response to 5 or 15 db pre-pulses (Figure 10B). The marble burying test measures the propensity of mice to engage repetitively in a natural digging behavior that is not confounded by anxiety (Thomas et al., 2009). MIA offspring displayed increased stereotyped marble burying compared to controls (Figure 10C), which models repetitive behavior as a core ASD symptom. Ultrasonic vocalizations are used to measure communication by mice, given that several types of calls are produced and used in structured motifs that vary across different social paradigms (Grimsley et al., 2011; Scattoni et al., 2011; Silverman et al., 2010b). MIA offspring exhibited ASD-related deficits in communication, as indicated by reduced number and duration of ultrasonic vocalizations produced in response to a social encounter (Figure 10D). Finally, the three-chamber social test is used to measure ASD-related impairments in social interaction (Silverman et al., 2010a). Sociability is exemplified by a mouse's preference to interact with a novel mouse over a novel object, while social novelty (social preference) is characterized by preference to interact with an unfamiliar versus a familiar mouse. MIA offspring exhibited deficits in both sociability and social preference (Figure 10E-F). Altogether, there behavioral assays evaluate the cardinal diagnostic symptoms of ASD, in addition to ASD-associated anxiety and deficient sensorimotor gating, have been broadly used to phenotype ASD mouse models (Han et al., 2012; Novarino et al., 2012; Schmeisser et al., 2012; Silverman et al., 2010a; Tabuchi et al., 2007; Tsai et al., 2012; Won et al., 2012).

[0140] Remarkably, oral treatment with *B. fragilis* ameliorated many of these ASD-related behavioral abnormalities. *B. fragilis*-treated MIA offspring did not exhibit anxiety-like behavior in the open field (Figure 10A; compare poly(I:C) (P) to poly(I:C)+*B. fragilis* (P+BF)), as shown by restoration in the number of center entries and duration of time spent in the center of the open field. *B. fragilis* improved sensorimotor gating in MIA offspring, as indicated by increased combined PPI in response to 5 and 15 db pre-pulses (Figure 10B), with no significant effect on the intensity of startle to the acoustic stimulus (data not shown). *B. fragilis*-treated MIA offspring also exhibited decreased levels of stereotyped marble burying and restored communicative behavior, as illustrated by increased

number and duration of ultrasonic vocalizations (Figure 10C-D). Interestingly, *B. fragilis* treatment raised the duration per call produced by MIA offspring to levels that exceed that observed in saline controls (Figure 10D), suggesting that despite normalization of the propensity to communicate (no difference compared to controls in the *number* of calls produced), there is a qualitative difference in the types of calls generated with enrichment of longer syllables.

[0141] Although *B. fragilis*-treated MIA offspring exhibited improved communicative, repetitive, anxiety-like and sensorimotor behavior, they retain deficits in sociability and social preference (Figure 10E). Interestingly, this parallels the inability to improve social behavior by administration of risperidone to ASD individuals (Canitano and Scandurra, 2008) and to CNTNAP2 knockout mice, a genetic mouse model for ASD (Penagarikano et al., 2011),. These data indicate that there are fundamental differences in the circuitry or circuit plasticity governing social behavior as compared to the other behaviors, and that *B. fragilis* treatment modulates specific brain circuits during amelioration of ASD-related behavioral defects in MIA offspring.

[0142] In addition, behavioral improvement in response to *B. fragilis* treatment was not associated with changes in systemic immunity in MIA offspring (Figure 3A-C) and was not dependent on polysaccharide A (PSA), a molecule previously identified to confer immunomodulatory effects by *B. fragilis* (Figure 3E) (Mazmanian et al., 2008; Ochoa-Reparaz et al., 2010; Round and Mazmanian, 2010). Furthermore, amelioration of behavior is not specific to *B. fragilis*, as similar treatment with *Bacteroides thetaiotaomicron*, also significantly improves anxiety-like, repetitive and communicative behavior in MIA offspring (Figure 11A-D). This is consistent with our finding that *B. fragilis* treatment does not lead to persistent colonization of *B. fragilis* in the GI tract (Figure 9A-B), and may be acting by causing long-term shifts in the resident microbiota (see Figure 4).

Example 6

The serum metabolome is modulated by MIA and *B. fragilis* treatment

[0143] Metabolomic studies have shown that gut microbial products are found in many extra-intestinal tissues, and molecules derived from the microbiota may influence metabolic, immunologic and behavioral phenotypes in mice and humans (Bercik et al., 2011;

Blumberg and Powrie, 2012; Hooper et al., 2012; MacFabe, 2012; Matsumoto et al., 2012; Nicholson et al., 2012). In this example, potential was examined.

[0144] Gas chromatography/liquid chromatography with mass spectrometry (GC/LC-MS)-based metabolomic profiling was used to identify MIA-associated changes in serum metabolites. 2,400 metabolites were assayed and of these, 322 metabolites, spanning amino acid (94), peptide (15), carbohydrate (22), energy (10), lipid (128), nucleotide (23), xenobiotic (19) and cofactor and vitamin (11) super pathways were detected in sera from adult mice (Table 4). Interestingly, MIA leads to statistically significant alterations in 8% of all serum metabolites detected (Table 3). Furthermore, postnatal *B. fragilis* treatment has a significant effect on the serum metabolome, altering 34% of all metabolites detected (Table 4 and Figure 12).

Table 3. Serum Metabolites Altered in Adult Saline versus Poly(I:C) Offspring

Super Pathway	Sub-pathway	Metabolite	Fold Change	*p*-value
Amino acid	Glycine, serine and threonine metabolism	N-acetylserine	0.73	0.0354
Amino acid	Alanine and aspartate metabolism	beta-alanine	0.46	0.0500
Amino acid	Glutamate metabolism	glutamine	1.2	0.0173
Amino acid	Histidine metabolism	transurocanate	1.71	0.0240
Amino acid	Histidine metabolism	imidazole propionate	1.35	0.0161
Amino acid	Phenylalanine and tyrosine metabolism	phenylacetylglycine	0.71	0.0821
Amino acid	Phenylalanine and tyrosine metabolism	phenol sulfate	0.68	0.0092
Amino acid	Tryptophan metabolism	indolepyruvate	1.57	0.0240
Amino acid	Tryptophan metabolism	serotonin	1.15	0.0804
Amino acid	Valine, leucine and isoleucine metabolism	3-methyl-2-oxovalerate	0.75	0.0152
Amino acid	Valine, leucine and isoleucine metabolism	4-methyl-2-oxopentaoate	0.7	0.0072

Amino acid	Cysteine, methionine, SAM, taurine metabolism	cysteine	0.73	0.0582
Amino acid	Urea cycle; arginine-, proline-, metabolism	arginine	0.87	0.0761
Amino acid	Urea cycle; arginine-, proline-, metabolism	ornithine	0.68	0.0956
Amino acid	Polyamine metabolism	5-methylthioadenosine	1.34	0.0425
Peptide	Dipeptide	glycylvaline	0.48	0.0077
Peptide	Fibrinogen cleavage peptide	TDTEDKGEFLSEGGGVR	1.8	0.0567
Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism	3-phosphoglycerate	0.51	0.0265
Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism	phosphoenolpyruvate	0.56	0.0344
Carbohydrate	Nucleotide sugars, pentose metabolism	ribose	1.44	0.0499
Carbohydrate	Nucleotide sugars, pentose metabolism	xylose	1.34	0.0827
Lipid	Essential fatty acid	docosapentaenoate (n3 DPA; 22:5n3)	0.75	0.0988
Lipid	Essential fatty acid	docosapentaenoate (n6 DPA; 22:5n6)	0.83	0.0970
Lipid	Essential fatty acid	docosahexaenoate (DHA; 22:6n3)	0.8	0.0965
Lipid	Long chain fatty acid	stearate	0.88	0.0491
Lipid	Long chain fatty acid	eicosenoate	0.61	0.0151
Lipid	Long chain fatty acid	dihomo-linoleate (20:2n6)	0.79	0.0614
Lipid	Long chain fatty acid	adrenate	0.82	0.0923
Lipid	Fatty acid, monohydroxy	13-HODE+9-HODE	0.72	0.0489
Lipid	Fatty acid, dicarboxylate	octadecanedioate	0.83	0.0413

Lipid	Eicosanoid	12-HETE	0.69	0.0152
Lipid	Inositol metabolism	myo-inositol	0.86	0.0817
Lipid	Lysolipid	1-palmitoylglycerophosphoethanolamine	0.81	0.0868
Lipid	Lysolipid	1-oleoylglycerophosphoethanolamine	0.7	0.0169
Lipid	Lysolipid	1-pentadecanoylglycerophosphocholine	1.43	0.0505
Lipid	Lysolipid	1-palmitoleoylglycerophosphocholine	1.49	0.0388
Lipid	Lysolipid	1-stearoylglycerophosphoinositol	0.64	0.0059
Lipid	Lysolipid	1-palmitoylplasmenylethanolamine	0.73	0.0399
Cofactors and vitamins	Hemoglobin and porphyrin metabolism	bilirubin (E,E)	2.68	0.0496
Cofactors and vitamins	Pantothenate and CoA metabolism	pantothenate	1.33	0.0643
Cofactors and vitamins	Benzoate metabolism	4-ethylphenylsulfate	46.39	0.0359
Cofactors and vitamins	Chemical	glycolate (hydroxyacetate)	1.17	0.0498
Cofactors and vitamins	Food component/Plant	ergothioneine	0.72	0.0688
Cofactors and vitamins	Food component/Plant	equol sulfate	0.78	0.0315

Summary of notable changes ($p<0.10$) in levels of serum metabolites in 10-week old offspring of poly(I:C)-injected mothers versus controls. Serum samples were extracted and analyzed by GC/LC-MS by Metabolon, Inc. Data were analyzed using two-way ANOVA with contrasts. Additional details are provided in Experimental Procedures.

Table 4. Serum Metabolites Altered in Saline and Poly(I:C) Offspring after *B. fragilis* Treatment

Super Pathway	Sub Pathway	Biochemical Name	Platform	I:C-Bfrag / CON
		sarcosine (N-Methylglycine)	GC/MS	0.64
	Alanine and aspartate metabolism	aspartate	GC/MS	0.76
		3-ureidopropionate	LC/MS pos	0.64
	Lysine metabolism	glutarate (pentanedioate)	GC/MS	0.78
		tyrosine	LC/MS pos	0.85
		3-(4-hydroxyphenyl)lactate	LC/MS neg	0.81
		3-phenylpropionate (hydrocinnamate)	LC/MS neg	0.60
		serotonin (5HT)	LC/MS pos	1.26
	Valine, leucine and isoleucine metabolism	3-methyl-2-oxobutyrate	LC/MS neg	0.68
		3-methyl-2-oxovalerate	LC/MS neg	0.67
		4-methyl-2-oxopentanoate	LC/MS neg	0.63
		isobutyrylcarnitine	LC/MS pos	0.68
		2-methylbutyroylcarnitine	LC/MS pos	0.66
		isovalerylcarnitine	LC/MS pos	0.76
		2-hydroxybutyrate (AHB)	GC/MS	0.64
	Urea cycle; arginine-,	arginine	LC/MS pos	0.86
		ornithine	GC/MS	0.66

	proline-, metabolism			
	Butanoate metabolism	2-aminobutyrate	LC/MS pos	0.76
	Guanidino and acetamido metabolism	4-guanidinobutanoate	LC/MS pos	0.65
		5-oxoproline	LC/MS neg	0.80
Peptide	Dipeptide	glycylvaline	LC/MS pos	0.22
		gamma-glutamyltryptophan	LC/MS pos	0.77
	Fibrinogen cleavage peptide	TDTEDKGEFLSEGGGV*	LC/MS pos	1.43
		TDTEDKGEFLSEGGGVR*	LC/MS pos	3.46
		sorbitol	GC/MS	0.63
		pyruvate	GC/MS	0.58
		ribitol	GC/MS	0.74
		ribose	GC/MS	1.97
		ribulose	GC/MS	0.68
		xylitol	GC/MS	1.62
Energy	Krebs cycle	citrate	GC/MS	0.80
		fumarate	GC/MS	0.64
		malate	GC/MS	0.69
Lipid	Essential fatty acid	linoleate (18:2n6)	LC/MS neg	0.64
		linolenate [alpha or gamma; (18:3n3 or 6)]	LC/MS neg	0.62
		dihomo-linolenate (20:3n3 or n6)	LC/MS neg	0.69
		docosapentaenoate (n3 DPA; 22:5n3)	LC/MS neg	0.72
		docosapentaenoate (n6 DPA; 22:5n6)	LC/MS neg	0.70
		docosahexaenoate (DHA; 22:6n3)	LC/MS neg	0.77
		heptanoate (7:0)	LC/MS neg	0.81
		pelargonate (9:0)	LC/MS neg	0.81
		laurate (12:0)	LC/MS neg	0.85
	Long chain fatty acid	myristate (14:0)	GC/MS	0.70
		palmitate (16:0)	LC/MS neg	0.72
		palmitoleate (16:1n7)	GC/MS	0.70
		margarate (17:0)	GC/MS	0.60
		stearate (18:0)	LC/MS neg	0.75
		oleate (18:1n9)	GC/MS	0.56
		stearidonate (18:4n3)	LC/MS neg	0.66
		eicosenoate (20:1n9 or 11)	LC/MS neg	0.59
		dihomo-linoleate (20:2n6)	LC/MS neg	0.63
		mead acid (20:3n9)	LC/MS neg	0.74
		adrenate (22:4n6)	LC/MS neg	0.75
		8-hydroxyoctanoate	LC/MS neg	0.72
		3-hydroxydecanoate	LC/MS neg	0.51
		16-hydroxypalmitate	LC/MS neg	0.70
		13-HODE + 9-HODE	LC/MS neg	0.50
	Fatty acid, dihydroxy	12,13-hydroxyoctadec-9(Z)-enoate	LC/MS neg	0.54
		9,10-hydroxyoctadec-12(Z)-enoic acid	LC/MS neg	0.48

	Fatty acid, dicarboxylate	adipate	GC/MS	0.62
		2-hydroxyglutarate	GC/MS	0.83
		pimelate (heptanedioate)	GC/MS	0.61
		suberate (octanedioate)	LC/MS pos	0.69
		sebacate (decanedioate)	LC/MS neg	0.64
		azelate (nonanedioate)	LC/MS neg	0.72
		dodecanedioate	LC/MS neg	0.65
		tetradecanedioate	LC/MS neg	0.57
		hexadecanedioate	LC/MS neg	0.54
		octadecanedioate	LC/MS neg	0.53
		undecanedioate	LC/MS neg	0.66
	Eicosanoid	12-HETE	LC/MS neg	0.57
	Fatty acid metabolism (also BCAA metabolism)	propionylcarnitine	LC/MS pos	0.79
		butyrylcarnitine	LC/MS pos	0.64
	Fatty acid metabolism	valerylcarnitine	LC/MS pos	0.56
		3-dehydrocarnitine*	LC/MS pos	0.71
		hexanoylcarnitine	LC/MS pos	0.58
		octanoylcarnitine	LC/MS pos	0.69
		choline	LC/MS pos	0.79
		chiro-inositol	GC/MS	0.66
		pinitol	GC/MS	0.61
	Ketone bodies	3-hydroxybutyrate (BHBA)	GC/MS	0.66
		1,2-propanediol	GC/MS	0.83
		1-linoleoylglycerophosphoethanolamine*	LC/MS neg	0.71
		1-arachidonoylglycerophosphoethanolamine*	LC/MS neg	0.76
		2-arachidonoylglycerophosphoethanolamine*	LC/MS neg	0.78
		1-stearoylglycerophosphoinositol	LC/MS neg	0.66
		1-linoleoylglycerophosphoinositol*	LC/MS neg	0.59
		1-arachidonoylglycerophosphoinositol*	LC/MS neg	0.61
		1-palmitoylplasmenylethanolamine*	LC/MS neg	0.72
		hypoxanthine	GC/MS	8.55
		inosine	LC/MS neg	8.36
		adenosine	LC/MS pos	5.63
		adenosine 5'-monophosphate (AMP)	LC/MS pos	20.92
	Purine metabolism, guanine containing	guanosine 5'- monophosphate (5'-GMP)	LC/MS pos	5.74
	Purine metabolism, urate metabolism	urate	LC/MS neg	0.84
		2'-deoxycytidine	LC/MS pos	1.32
	Pyrimidine metabolism, uracil containing	uracil	GC/MS	0.64
		pseudouridine	LC/MS neg	0.89

	Nicotinate and nicotinamide metabolism	nicotinamide	LC/MS pos	0.79
		catechol sulfate	LC/MS neg	0.77
	Drug	salicylate	LC/MS neg	0.68
		equol sulfate	LC/MS neg	0.70
	Sugar, sugar substitute, starch	erythritol	GC/MS	0.79

Example 7

B. fragilis treatment corrects levels of MIA-induced serum metabolites

[0145] This examples shows *B. fragilis*-mediated improvement of intestinal barrier integrity prevents alterations in serum metabolite levels.

[0146] 4-ethylphenylsulfate (4EPS), indolepyruvate and several other serum metabolites are significantly altered by MIA treatment and restored to control levels by *B. fragilis* treatment (Figure 13A). MIA offspring displayed a striking, 46-fold increase in serum levels of 4-ethylphenylsulfate (4EPS) which was dramatically reduced by *B. fragilis* treatment (Figure 13A). Moreover, it was found that compared to conventionally colonized mice, germ-free mice display nearly undetectable levels of serum 4EPS, indicating that serum 4EPS is derived from, or critically modulated by, the commensal microbiota (Figure 13B). 4EPS has been suggested to be a uremic toxin, as is *p*-cresol (4-methylphenol), a related metabolite identified as a possible urinary biomarker for human autism (Altieri et al., 2011; Persico and Napolioni, 2013). MIA offspring also exhibited elevated levels of serum *p*-cresol, although the increase did not reach statistical significance (Table 4). The fact that 4EPS shares close structural similarity to the toxic sulfated form of *p*-cresol (4-methylphenylsulfate; 4MPS) is intriguing as the two metabolites may exhibit functional overlap (Figure 14A) and link metabolite abnormalities seen in the MIA model to those observed in human ASD.

[0147] In addition to 4EPS, MIA offspring displayed significantly increased levels of serum indolepyruvate, a key molecule of the tryptophan metabolism pathway, which was restored to control levels by *B. fragilis* treatment (Figure 13A). Indolepyruvate is generated by tryptophan catabolism and, like 4EPS, indolepyruvate is believed to be

produced by gut microbes (Smith and Macfarlane, 1997) (Figure 14B). Moreover, the elevation in serum indolepyruvate observed in MIA offspring is analogous to the increase in another major tryptophan metabolite observed in human autism, indolyl-3-acryloylglycine (IAG), which was suggested to be a urinary biomarker for ASD (Bull et al., 2003). Interestingly, IAG is involved in GI homeostasis and is produced by bacterial tryptophan metabolism (Keszthelyi et al., 2009). It is notable that MIA offspring exhibited increased levels of serum serotonin ($0.05 < P < 0.10$), which reflects an alteration in another pathway of tryptophan metabolism and is reminiscent of the hyperserotonemia endophenotype of autism (Mulder et al., 2004). Importantly, the commensal microbiota is known to impact serum levels of indole-containing tryptophan metabolites and serotonin (Wikoff et al., 2009). MIA also led to altered serum glycolate, imidazole propionate and N-acetylserine levels (Figure 13A), which were corrected by *B. fragilis* treatment.

[0148] This example demonstrates that specific metabolites are altered in MIA offspring and normalized by *B. fragilis* treatment.

Example 8

A serum metabolite induces ASD-related behaviors

[0149] MIA-dependent increases in the systemic bioavailability of specific metabolites, and restoration by *B. fragilis*, suggest that these molecules play a causative role in ASD-related behaviors in MIA offspring. This example examined whether experimentally increasing serum 4EPS, the most dramatic of all metabolites affected by gut bacteria, is sufficient to cause any ASD-related behavioral abnormalities in naïve mice.

[0150] 4EPS was chemically synthesized by treatment of 4-ethylphenol with sulfur trioxide-pyridine complex, which, following ion exchange, yields 4EPS potassium salt (Figures 15A-C) (Burlingham et al., 2003; Grimes, 1959). Mice were intraperitoneally treated with 4EPS or saline vehicle daily, from 3 weeks of age (when increased gut permeability was detected in MIA offspring, see Figure 1A) to 6 weeks of age (when behavior testing began).

[0151] Remarkably, systemic administration of 4EPS to naïve wild-type mice was sufficient to induce anxiety-like behavior similar to that observed in MIA offspring (Figure 13C). Relative to vehicle-treated controls, mice exposed to 4EPS traveled comparable distances in the open field but spent less time in the center arena (Figure 13C).

Notably, vehicle-treated controls exhibited symptoms of anxiety-like behavior compared to untreated saline offspring (center entries: 14.5 ± 1.1 versus 23.7 ± 1.4; center duration (s): 29.4 ± 5.4 versus 46.4 ± 4.2; distance (m): 35.6 ± 1.8 versus 37.6 ± 1.0, comparing vehicle-treated mice (Veh.) in Figure 13C to saline offspring (S) in Figure 10A). This reflects the well-known effect of chronic stress (daily injection) on raising anxiety levels in mice and humans (Bailey and Crawley, 2009; Bourin et al., 2007). Also, in the PPI test, 4EPS-treated mice exhibited increased intensity of startle in response to the unconditioned primary stimulus, but no significant alterations in PPI (Figure 13D), representing anxiety-associated potentiation of the startle reflex (Bourin et al., 2007). Also, there was no difference in weight between 4EPS- and control-treated mice, and thus, no confounding effect of body mass on measured startle intensity. Conversely, there were no significant differences between 4EPS-treated versus saline-treated mice in marble burying or USV behavior (Figures 15D and 15E), suggesting that elevating serum 4EPS levels specifically promoted anxiety-like behavior.

Example 9

Treatment of Autism Spectrum Disorder (ASD)

[0152] This example illustrates the treatment of a patient suffering from ASD.

[0153] A patient is identified as being suffering from ASD. The blood level of 4EPS in the subject is determined. A composition with *B. fragilis* is administered to the patient via oral administration. The administration of *B. fragilis* is expected to alter the blood level of 4EPS and composition of gut microbiota in the patient. It is also expected that the bacterial administration will relieve one or more symptoms of ASD, such as improve behavioral performance, in the patient.

Example 10

Treatment of Autism Spectrum Disorder (ASD)

[0154] This example illustrates the treatment of a patient suffering from ASD.

[0155] A patient is identified as being suffering from ASD. The urine level of 4-methylphenysulfate in the subject is determined. A composition with *B. fragilis* is administered to the patient via oral administration. The administration of *B. fragilis* is expected to alter the urine level of 4-methylphenysulfate and the composition of gut

microbiota in the patient. It is also expect that the bacterial administration will relieve one or more symptoms of ASD, such as improve behavioral performance, in the patient.

[0156] The foregoing description and examples detail certain preferred embodiments of the invention and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof. Although the present application has been described in detail above, it will be understood by one of ordinary skill in the art that various modifications can be made without departing from the spirit of the invention.

[0157] In this application, the use of the singular can include the plural unless specifically stated otherwise or unless, as will be understood by one of skill in the art in light of the present disclosure, the singular is the only functional embodiment. Thus, for example, “a” can mean more than one, and "one embodiment" can mean that the description applies to multiple embodiments. Additionally, in this application, “and/or” denotes that both the inclusive meaning of "and" and, alternatively, the exclusive meaning of "or" applies to the list. Thus, the listing should be read to include all possible combinations of the items of the list and to also include each item, exclusively, from the other items. The addition of this term is not meant to denote any particular meaning to the use of the terms “and” or “or” alone. The meaning of such terms will be evident to one of skill in the art upon reading the particular disclosure.

[0158] All references cited herein including, but not limited to, published and unpublished patent applications, patents, text books, literature references, and the like, to the extent that they are not already, are hereby incorporated by reference in their entirety. To the extent that one or more of the incorporated literature and similar materials differ from or contradict the disclosure contained in the specification, including but not limited to defined terms, term usage, described techniques, or the like, the specification is intended to supersede and/or take precedence over any such contradictory material.

[0159] The term "comprising" as used herein is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps.

2013308826 19 Mar 2019

WHAT IS CLAIMED IS:

1. A method for improving behavioral performance in a subject suffering from anxiety, Fragile X, Rett syndrome, tuberous sclerosis, obsessive compulsive disorder, attention deficit disorder, schizophrenia, autistic disorder (classic autism), Asperger's disorder (Asperger syndrome), pervasive developmental disorder not otherwise specified (PDD-NOS), or childhood disintegrative disorder (CDD), the method comprising:

determining the level of 4-ethylphenylsulfate in the blood of a subject in need of treatment, wherein said level of 4-ethylphenylsulfate in the subject is altered relative to a reference level of 4-ethylphenylsulfate observed in a subject not suffering from or showing symptoms of anxiety, Fragile X, Rett syndrome, tuberous sclerosis, obsessive compulsive disorder, attention deficit disorder, schizophrenia, autistic disorder (classic autism), Asperger's disorder (Asperger syndrome), PDD-NOS, or CDD; and

administering a composition comprising *Bacteroides* bacteria tothe subject until an improvement in the behavioral performance in the subject is observed.

2. The method of claim 1, wherein the composition comprising *Bacteroides* bacteria is a probiotic composition, a nutraceutical composition, a pharmaceutical composition, or a mixture thereof.

3. The method of claim 1 or claim 2, wherein administering to the subject a composition comprising *Bacteroides* bacteria is performed via oral administration, rectal administration, transdermal administration, intranasal administration, or inhalation.

4. The method of any one of claims 1 to 3, wherein the *Bacteroides* bacteria is *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatis*, or a mixture thereof.

5. The method of any one of claims 1 to 4, wherein the *Bacteroides* bacteria comprises *B. fragilis*.

2013308826 19 Mar 2019

6. The method of any one of claims 1 to 5, wherein administering to the subject a composition comprising *Bacteroides* bacteria comprises fecal transplantation.

7. The method of any one of claims 1 to 6, the method further comprising administering to the subject an antibody against the 4-ethylphenylsulfate, an antibody against an intermediate for the in vivo synthesis of the 4-ethylphenylsulfate, or an antibody against a substrate for the in vivo synthesis of the 4-ethylphenylsulfate.

8. The method of any one of claims 1 to 7, the method further comprising inhibiting an enzyme involved in the in vivo synthesis of the 4-ethylphenylsulfate.

9. The method of any one of claims 1 to 8, further comprising administering a reduced carbohydrate diet to the subject.

10. The method of any one of claims 1 to 9, wherein the subject in need of treatment suffers from at least one of autistic disorder, Asperger's disorder, PDD-NOS, Rett syndrome, and CDD.

11. The method of any one of claims 1 to 10, wherein the subject in need of treatment suffers from anxiety.

12. The method of any one of claims 1 to 11, wherein the subject further suffers from gastrointestinal distress, wherein administering the composition ameliorates the gastrointestinal distress.

13. The method of claim 12, wherein the gastrointestinal distress comprises abdominal cramps, chronic diarrhea, constipation, intestinal permeability, or a combination thereof.

14. The method of any one of claims 1 to 13, wherein the behavioral performance comprises one or more of locomotion behavior, anxiety-like behavior, sensorimotor behavior, communicative behavior, sociability, social preference, repetitive behavior, and stereotyped behavior.

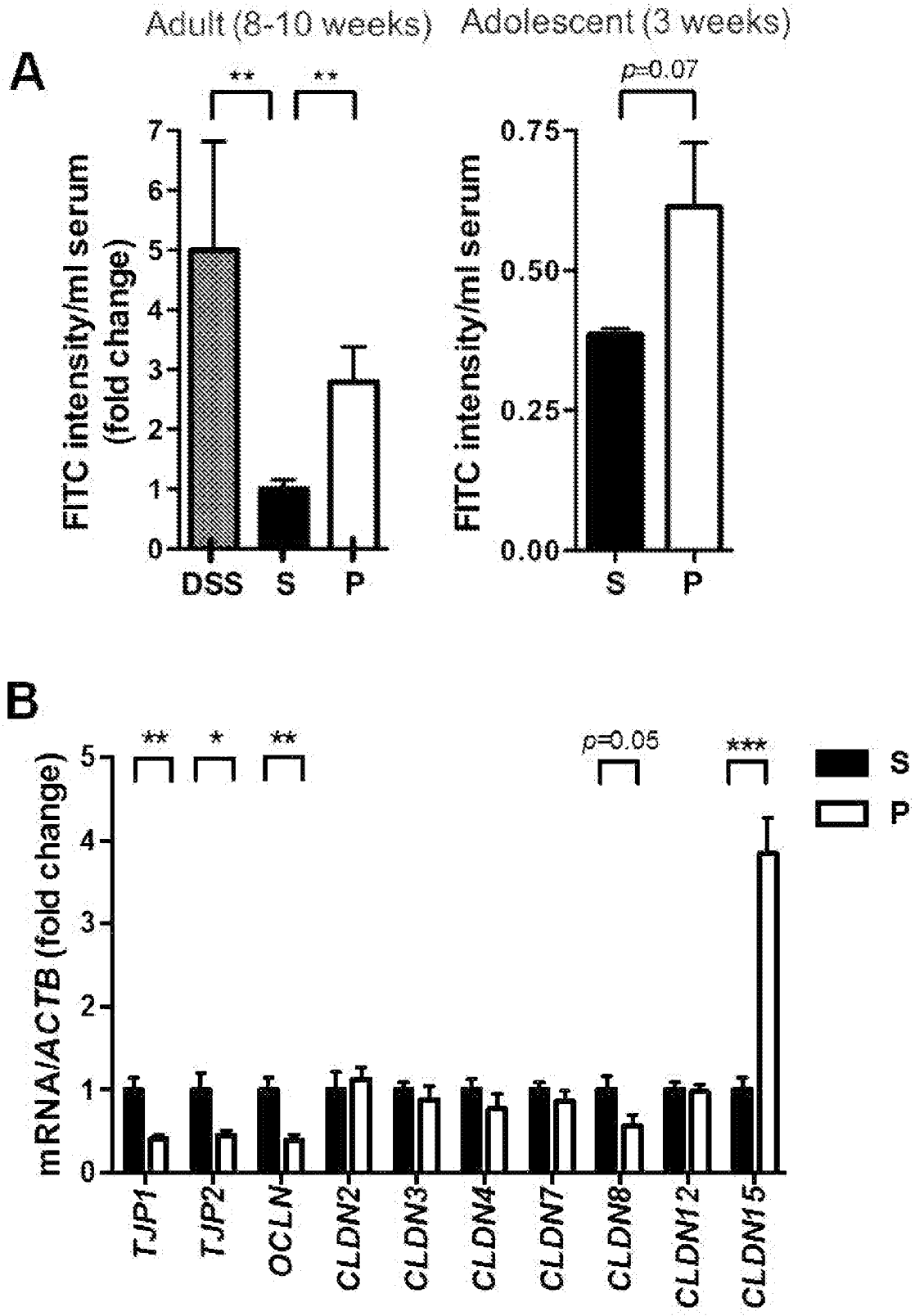
2013308826 19 Mar 2019

15. The method of any one of claims 1 to 14, comprising determining at least one of the one or more behavior features of the subject prior to and/or after adjusting the blood level of the 4-ethylphenylsulfate in the subject.

16. The method of claim 15, wherein determining at least one of the one or more behavior features of the subject comprises using one or more of Autism Behavior Checklist (ABC), Autism diagnostic Interview-Revised (ADI-R), childhood autism Rating Scale (CARS), and Pre-Linguistic Autism Diagnostic Observation Schedule (PL-ADOS).

17. The method of any one of claims 1 to 16, further comprising administering one or more of *Ruminococcaceae, Erysipelotrichaceae*, and *Alcaligenaceae* bacteria to the subject.

Figure 1


A
Adult (8-10 weeks)
Adolescent (3 weeks)
FITC intensity/ml serum (fold change)
FITC intensity/ml serum
**
**
p=0.07
DSS
S
P
S
P
B
mRNA/ACTB (fold change)
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*
**
p=0.05
***
S
P
TJP1
TJP2
OCLN
CLDN2
CLDN3
CLDN4
CLDN7
CLDN8
CLDN12
CLDN15

Figure 1 (continued)

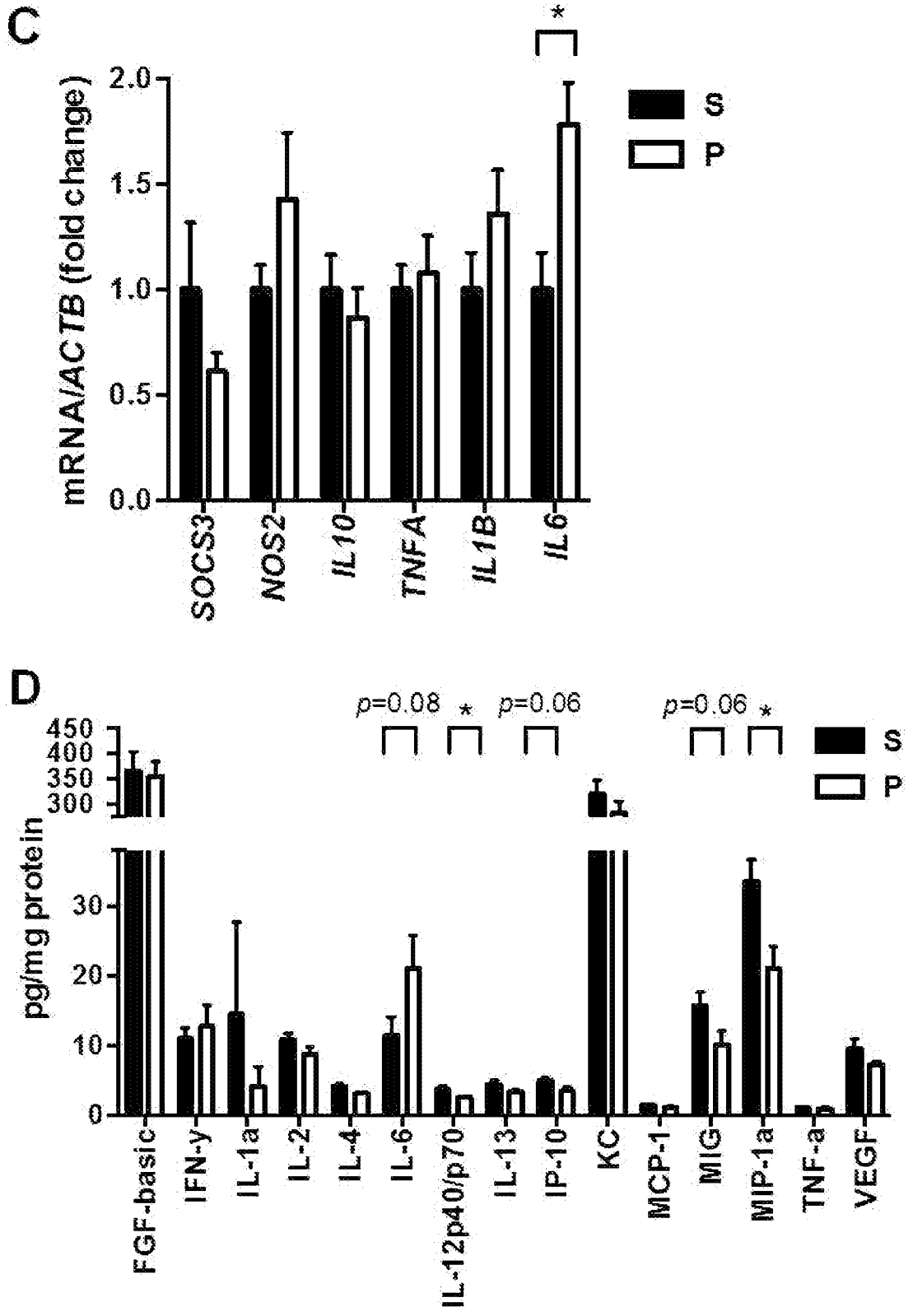


Figure 2

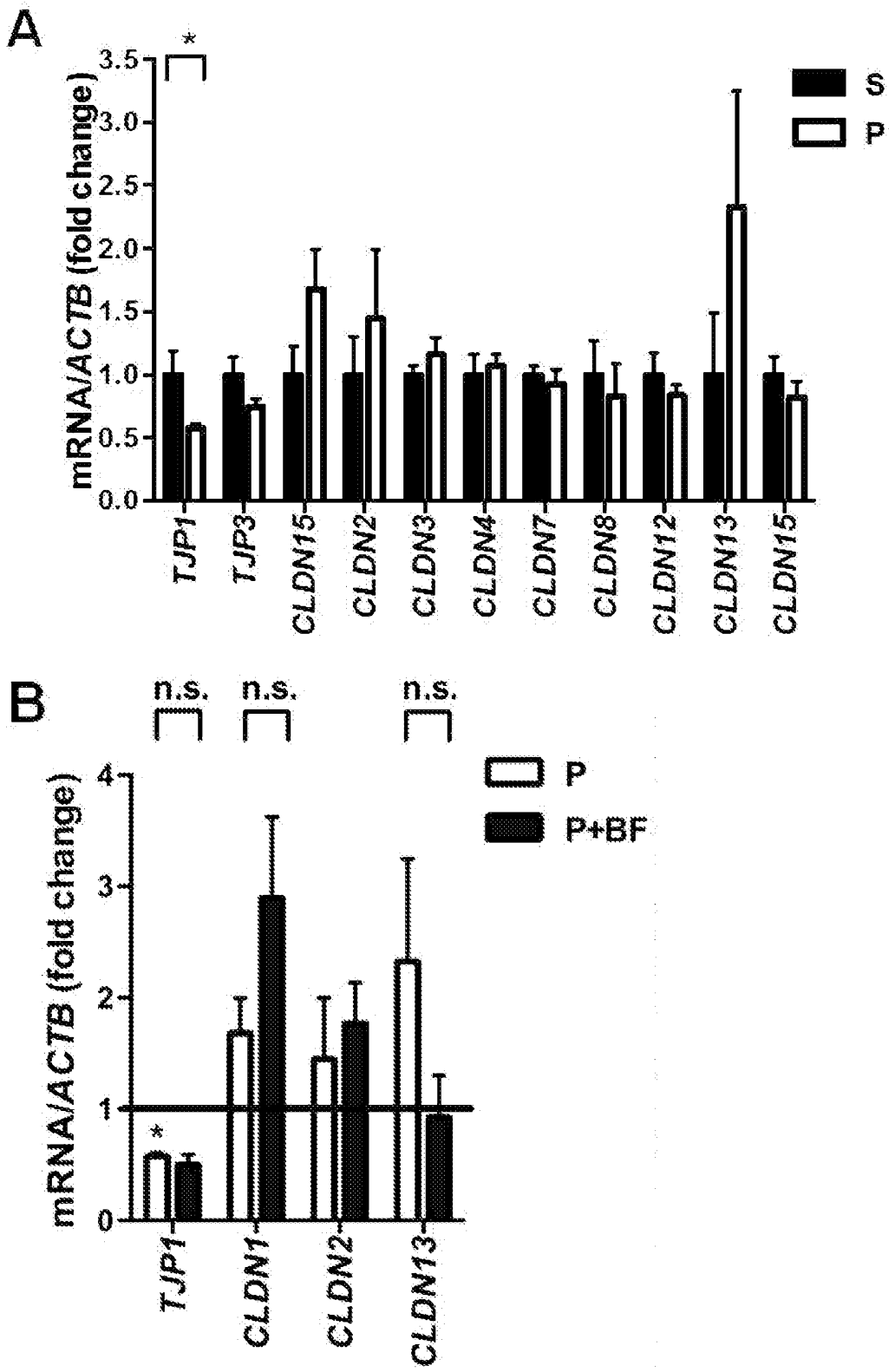

A
*
S
P
mRNA/ACTB (fold change)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TJP1
TJP3
CLDN15
CLDN2
CLDN3
CLDN4
CLDN7
CLDN8
CLDN12
CLDN13
CLDN15
B
n.s.
n.s.
n.s.
P
P+BF
mRNA/ACTB (fold change)
4
3
2
1
0
*
TJP1
CLDN1
CLDN2
CLDN13

Figure 2 (continued)

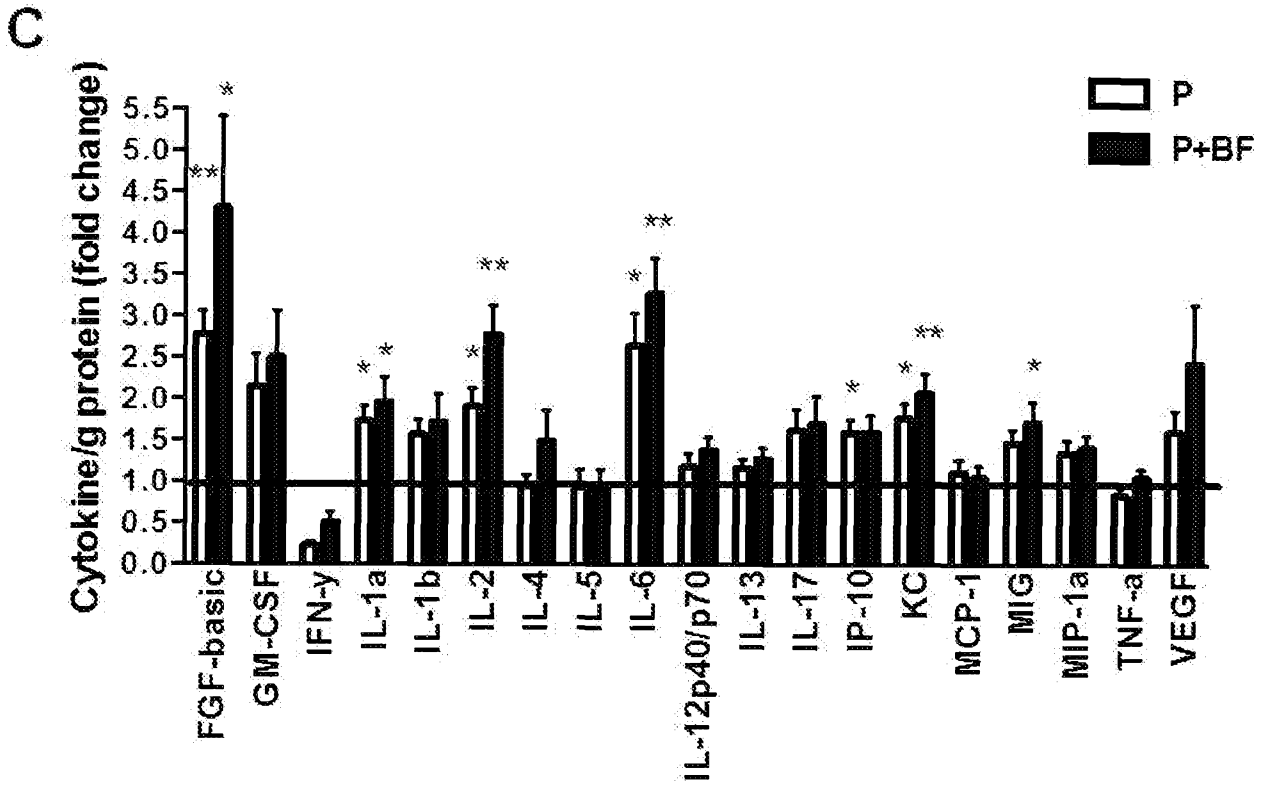


Figure 3

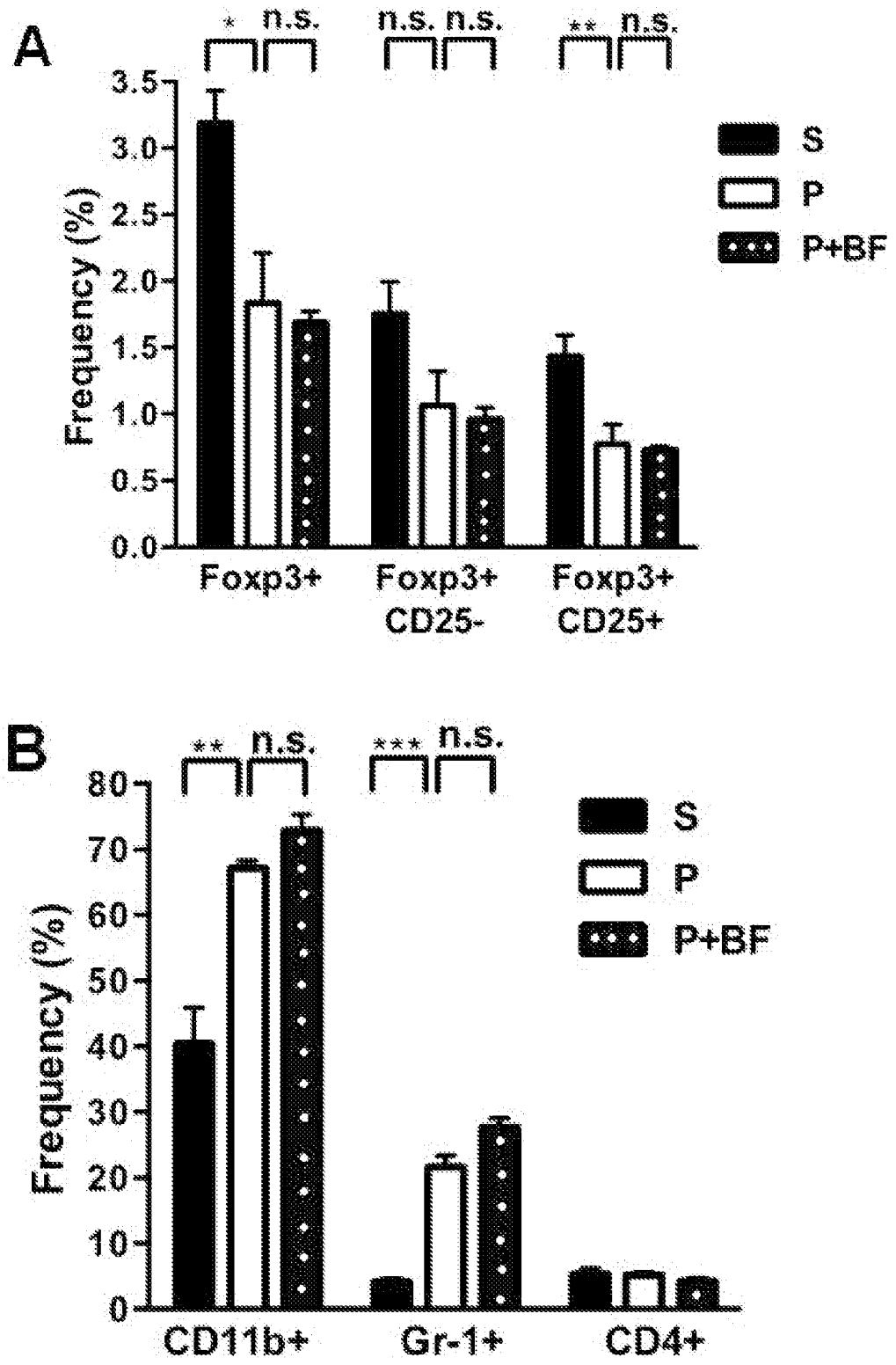

A
*
n.s.
n.s.
n.s.
**
n.s.
Frequency (%)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
S
P
P+BF
Foxp3+
Foxp3+ CD25-
Foxp3+ CD25+
B
**
n.s.
***
n.s.
Frequency (%)
80
70
60
50
40
30
20
10
0
S
P
P+BF
CD11b+
Gr-1+
CD4+

Figure 3 (continued)

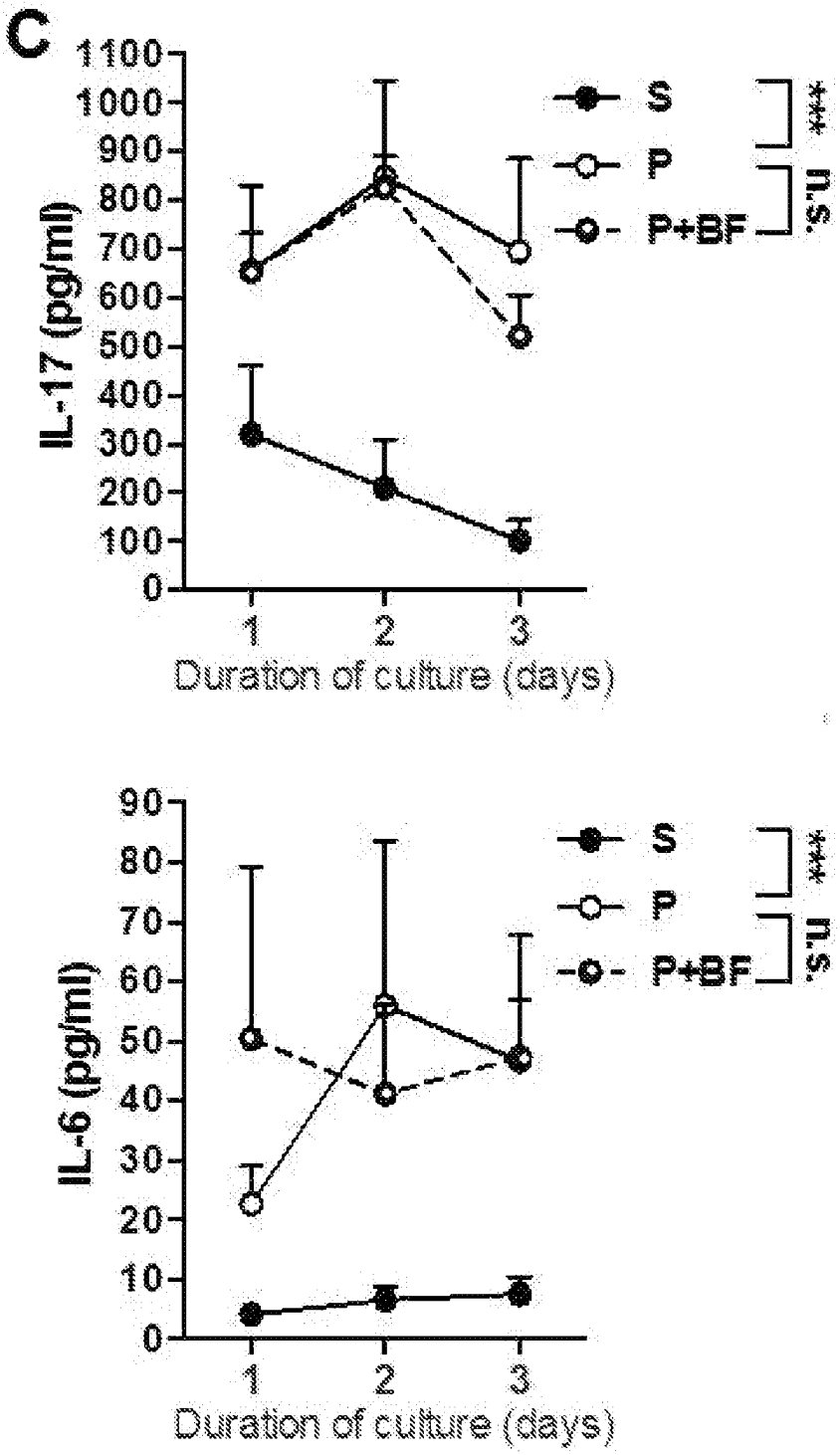


Figure 3 (continued)

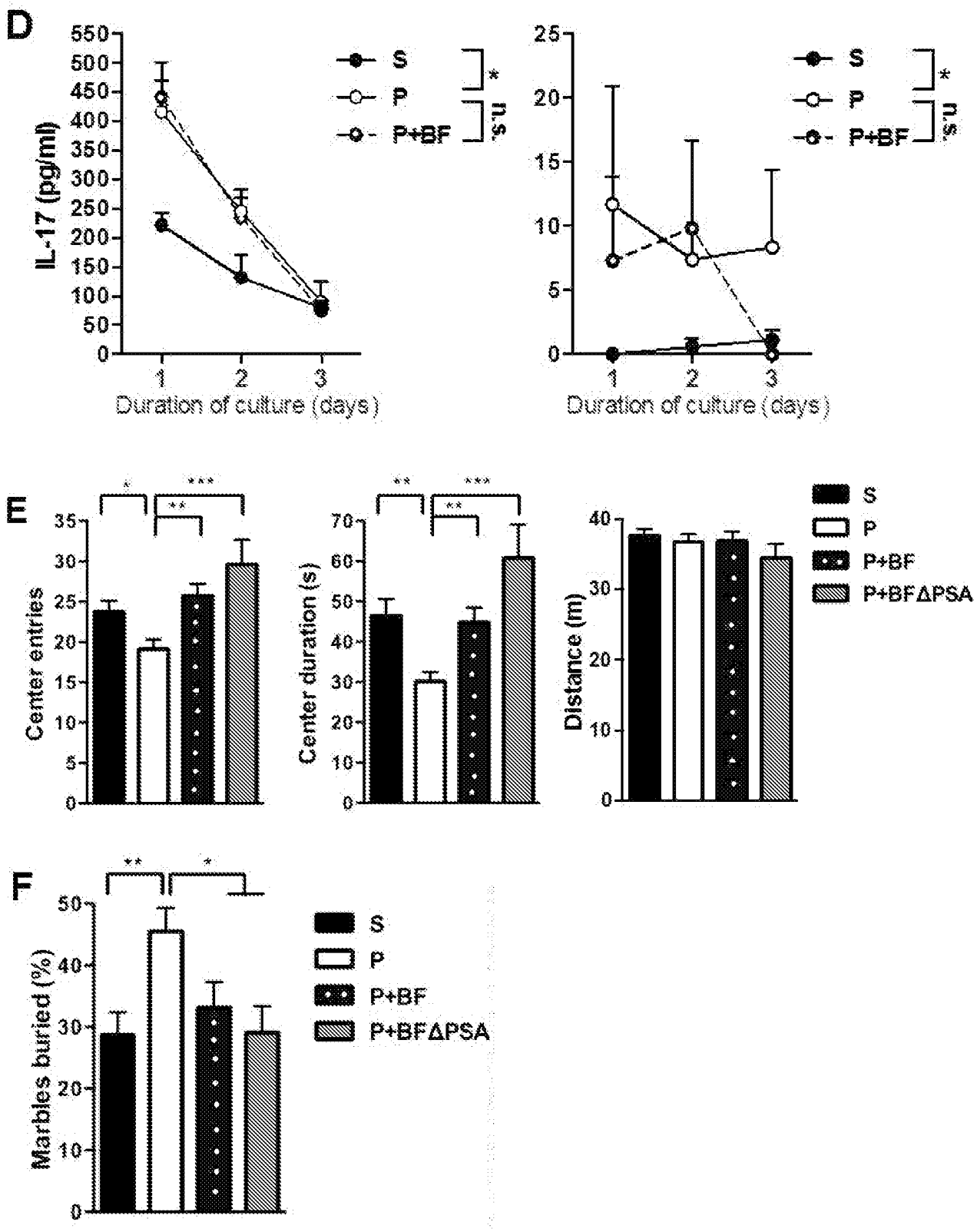


Figure 4

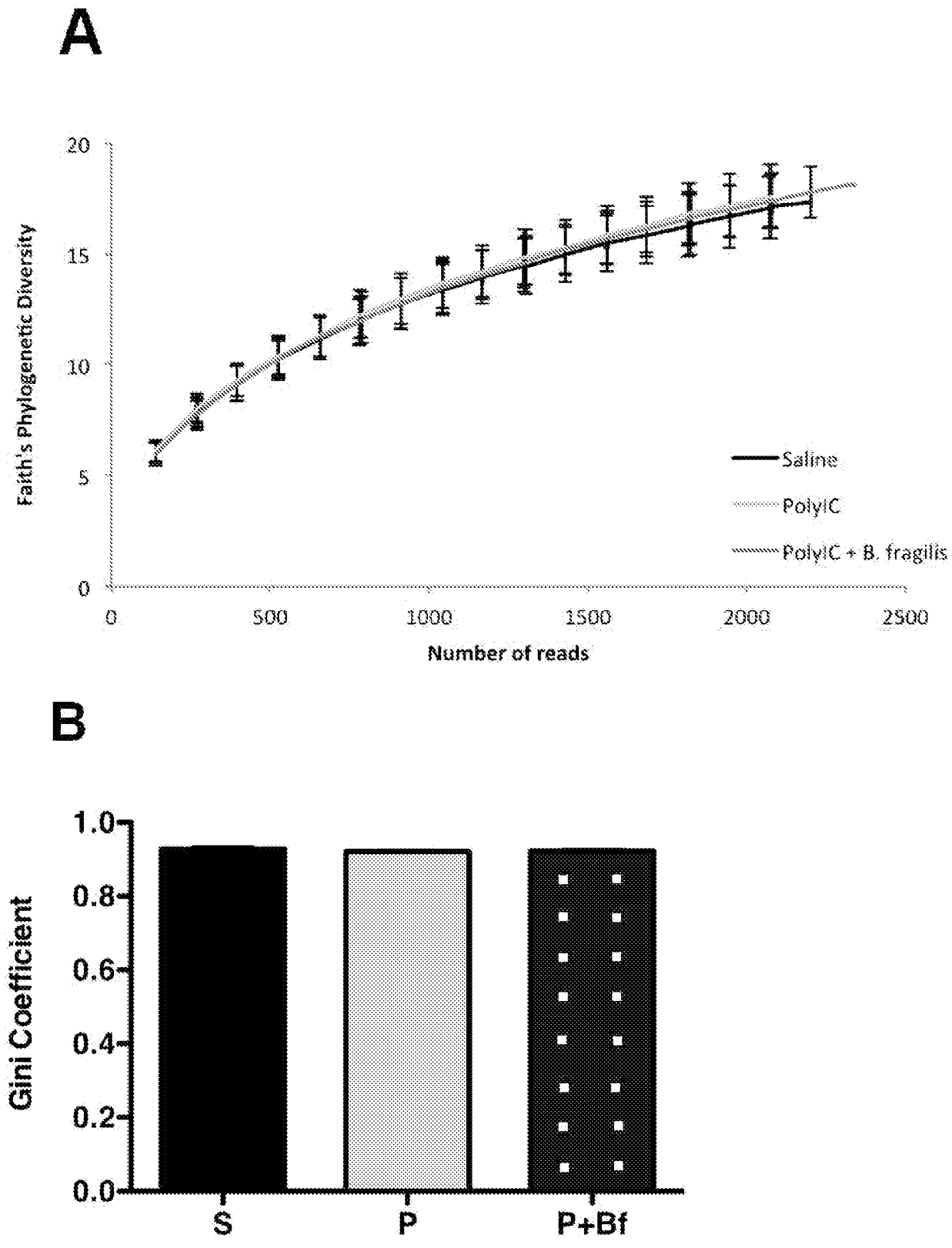

A
Faith's Phylogenetic Diversity
20
15
10
5
0
0
500
1000
1500
2000
2500
Number of reads
Saline
PolyIC
PolyIC + B. fragilis
B
Gini Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
S
P
P+Bf

Figure 4 (continued)

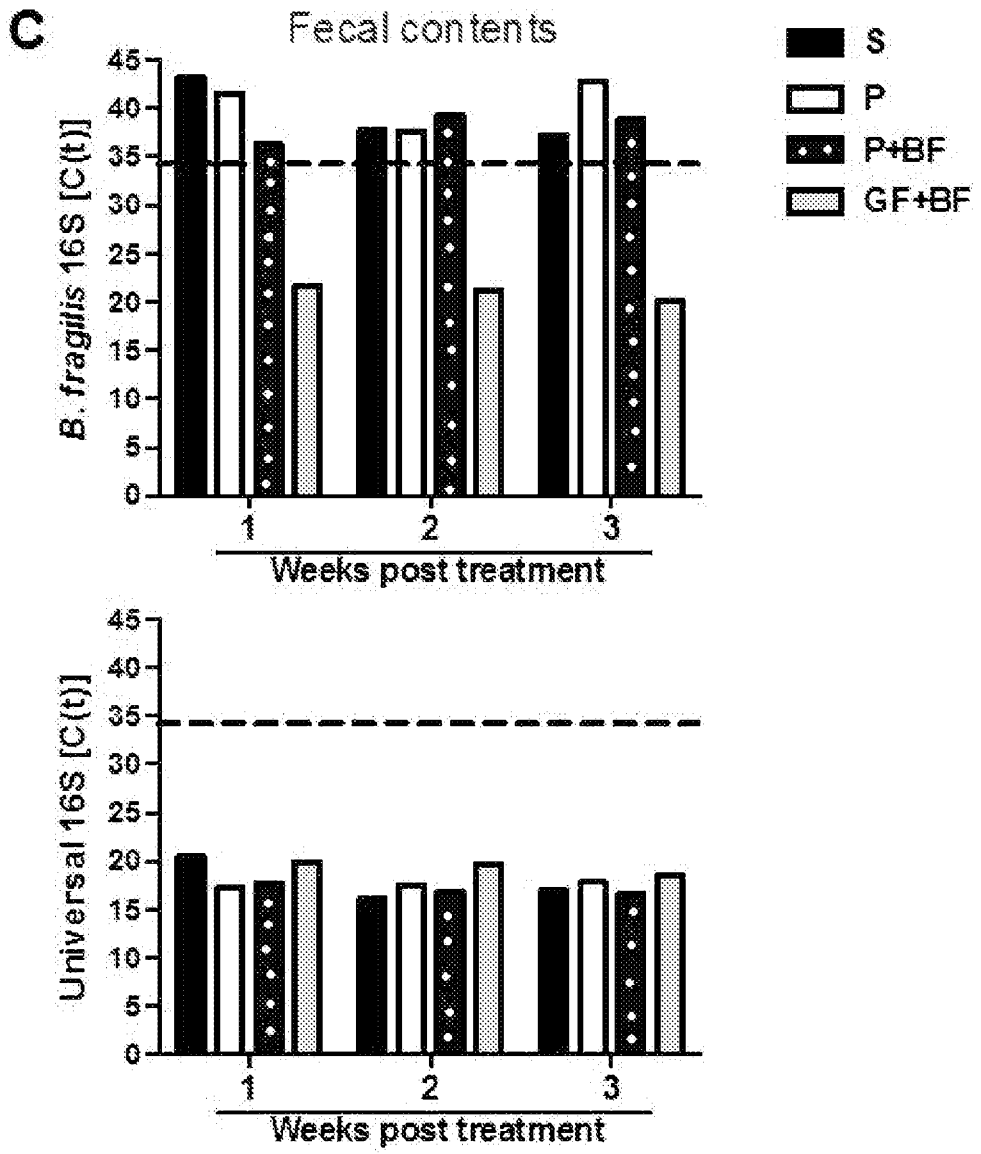


Figure 4 (continued)

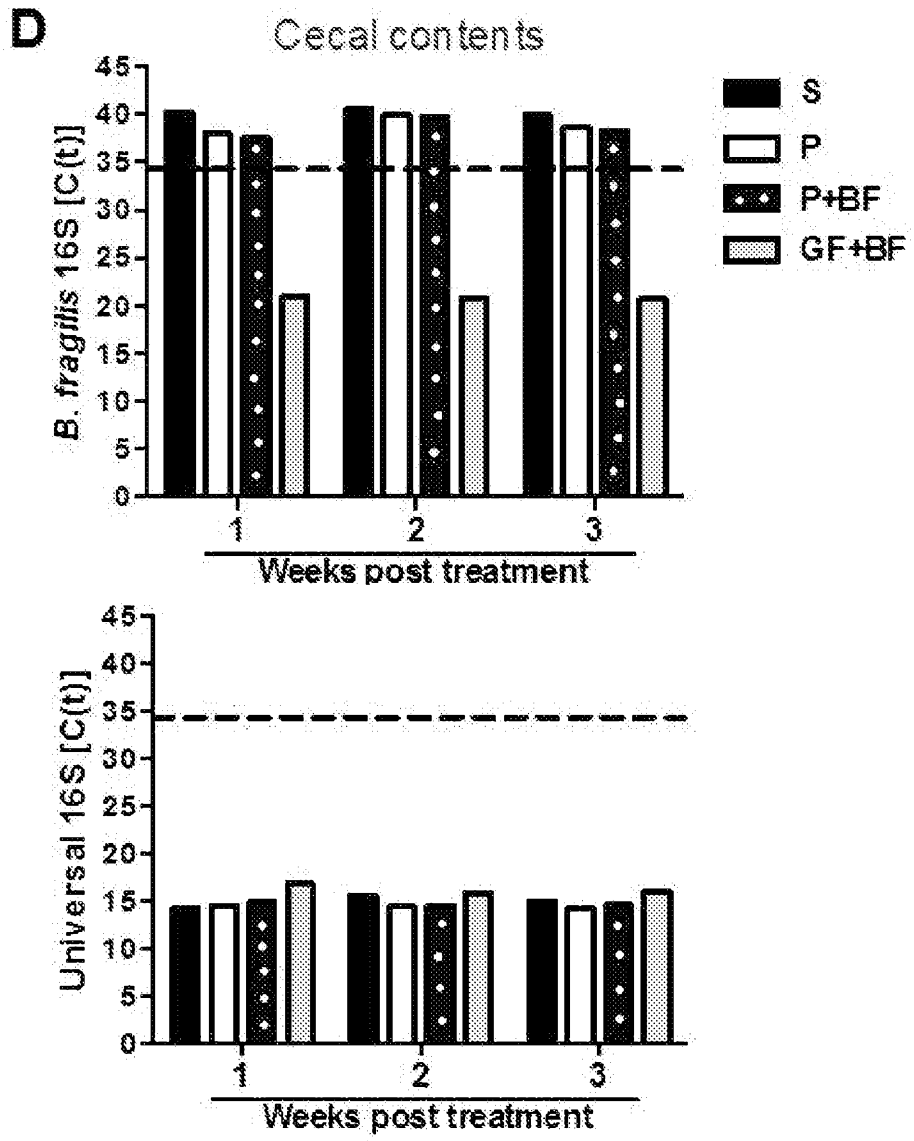


Figure 5A

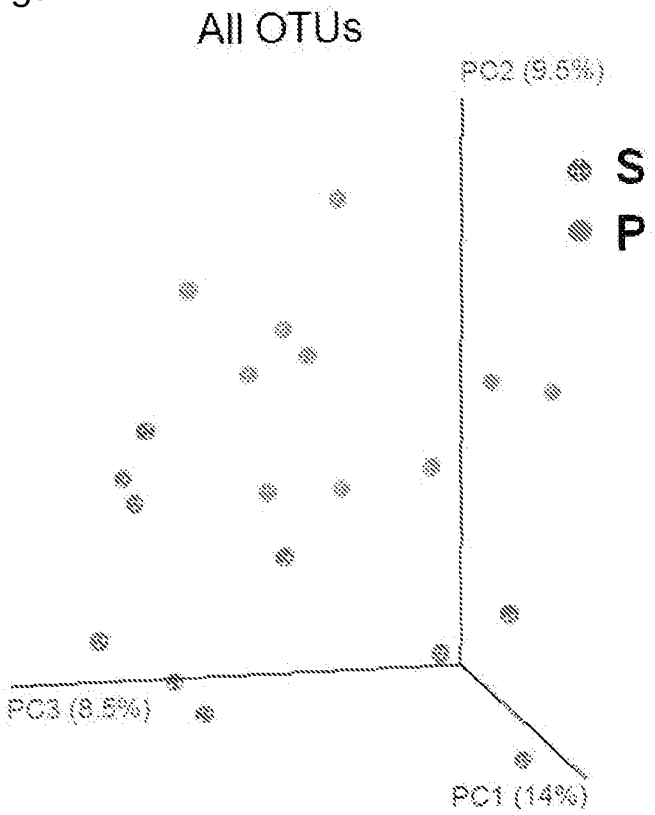


Figure 5B

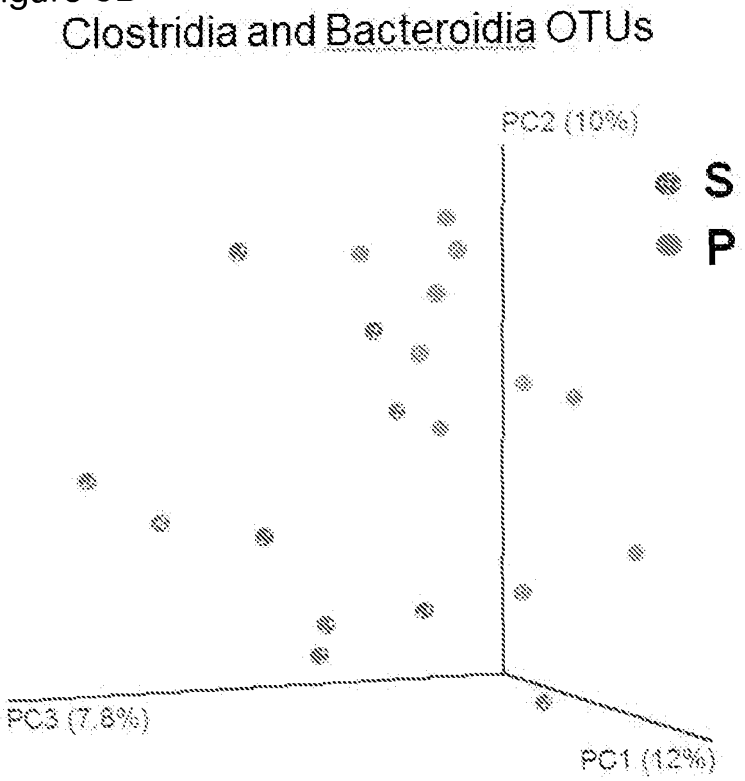
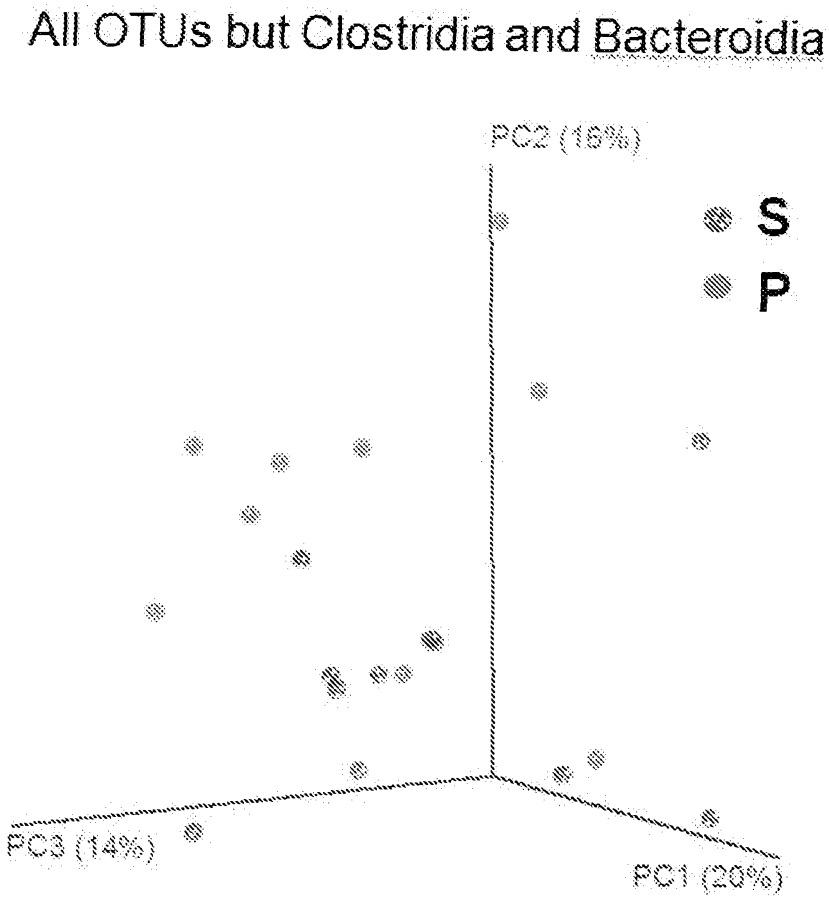


Figure 5C


All OTUs but Clostridia and Bacteroidia
PC2 (16%)
S
P
PC3 (14%)
PC1 (20%)

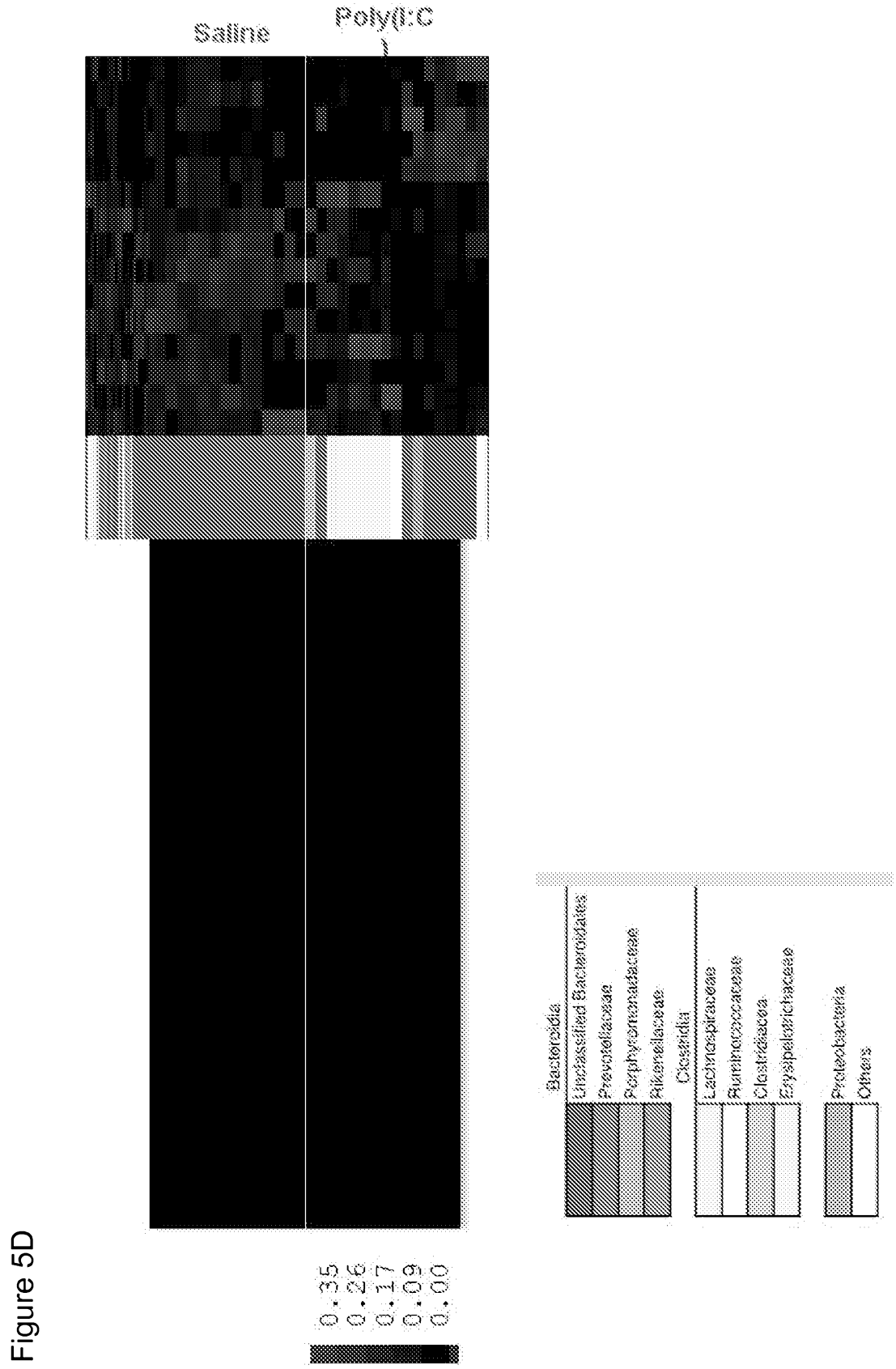

Saline
Poly(I:C)
0.35
0.26
0.17
0.09
0.00
Bacteroidia
Unclassified Bacteroidales
Prevotellaceae
Porphyromonadaceae
Rikenellaceae
Clostridia
Lachnospiraceae
Ruminococcaceae
Clostridiaceae
Erysipelotrichaceae
Proteobacteria
Others

Figure 5D

Figure 5E

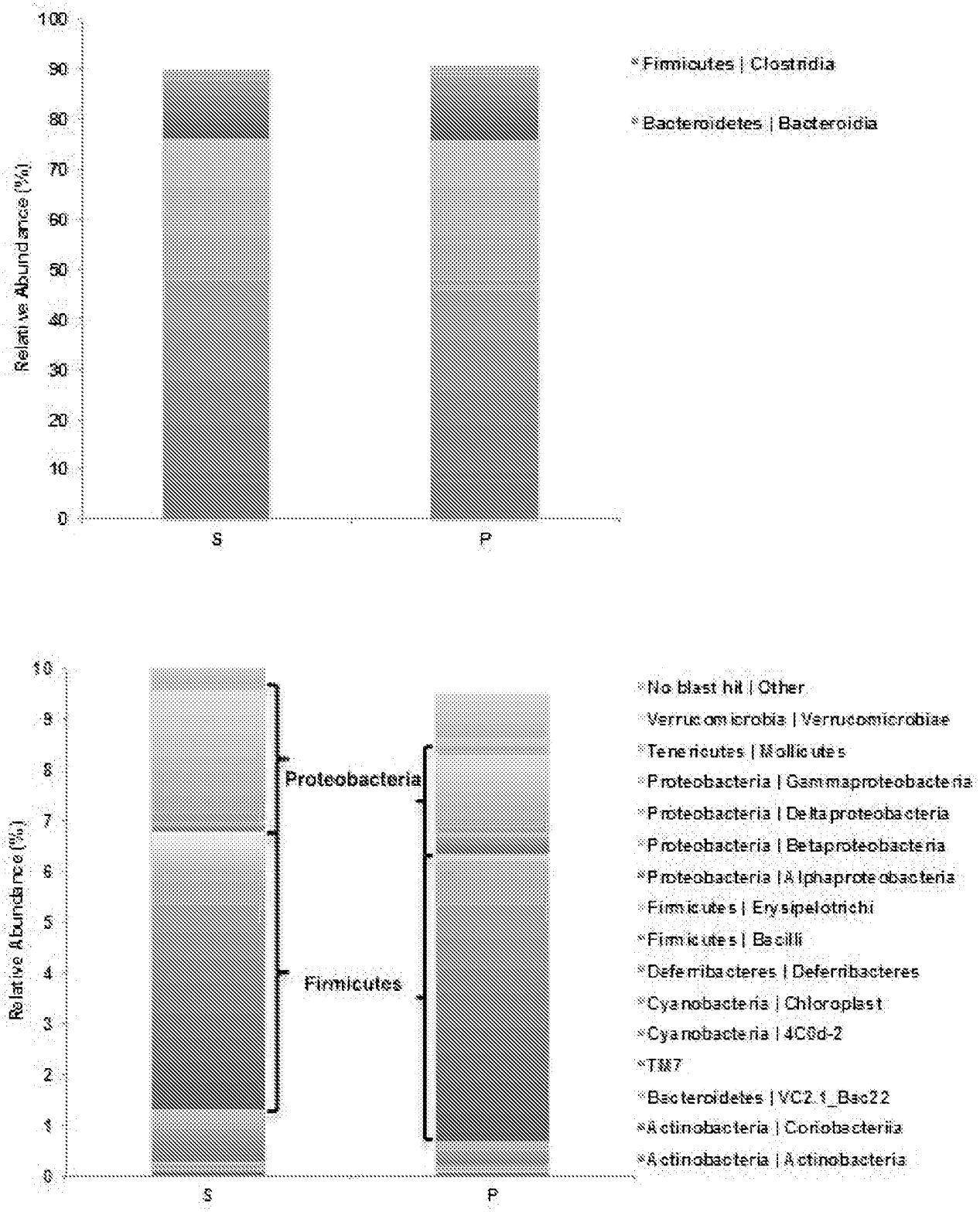

Relative Abundance (%)
100
90
80
70
60
50
40
30
20
10
0
S
P
Firmicutes | Clostridia
Bacteroidetes | Bacteroidia
Proteobacteria
Firmicutes
No blast hit | Other
Verrucomicrobia | Verrucomicrobiae
Tenericutes | Mollicutes
Proteobacteria | Gammaproteobacteria
Proteobacteria | Deltaproteobacteria
Proteobacteria | Betaproteobacteria
Proteobacteria | Alphaproteobacteria
Firmicutes | Erysipelotrichi
Firmicutes | Bacilli
Deferribacteres | Deferribacteres
Cyanobacteria | Chloroplast
Cyanobacteria | 4C0d-2
TM7
Bacteroidetes | VC2.1_Bac22
Actinobacteria | Coriobacteriia
Actinobacteria | Actinobacteria

Figure 6

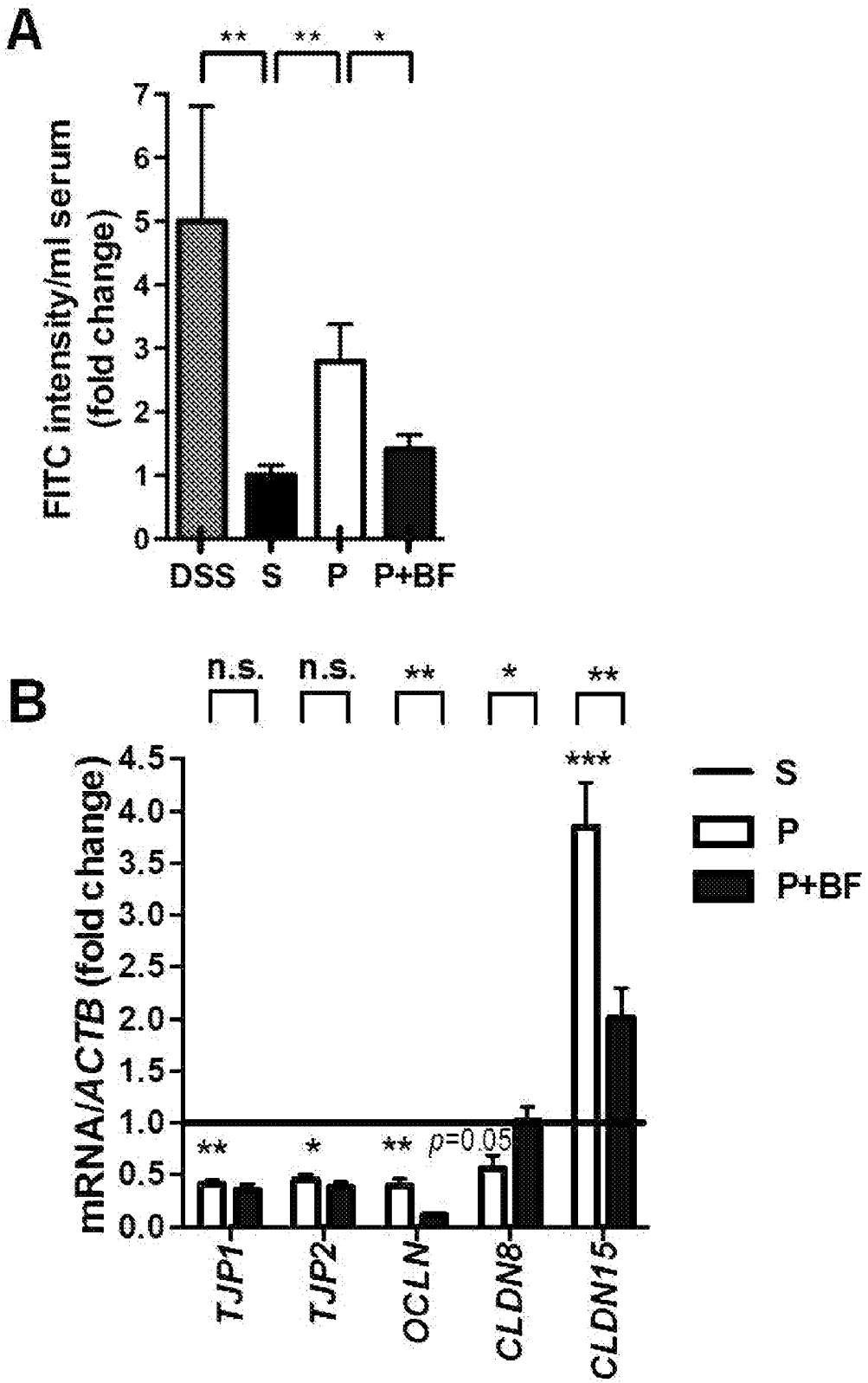

A
**
**
*
FITC intensity/ml serum (fold change)
0
1
2
3
4
5
6
7
DSS
S
P
P+BF
B
n.s.
n.s.
**
*
**
***
mRNA/ACTB (fold change)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
**
*
** p=0.05
TJP1
TJP2
OCLN
CLDN8
CLDN15
S
P
P+BF

Figure 6 (continued)

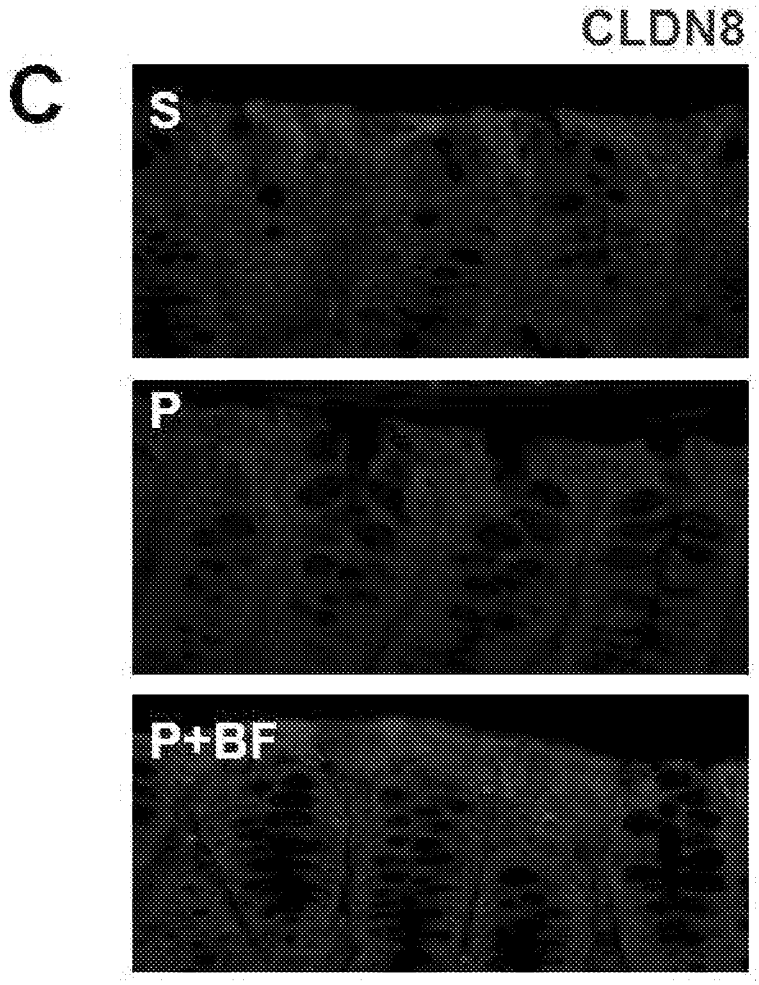


Figure 6 (continued)

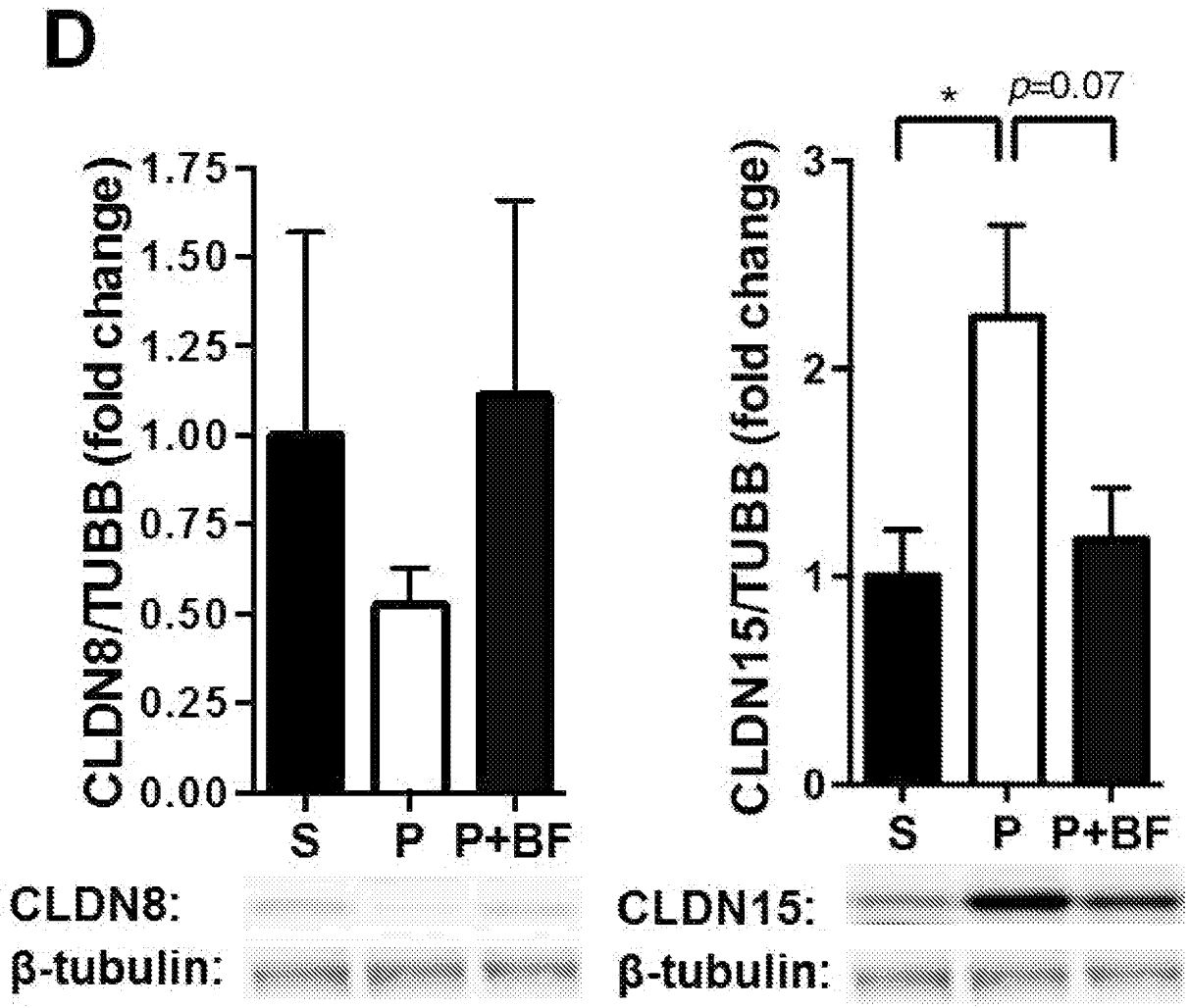


Figure 6 (continued)

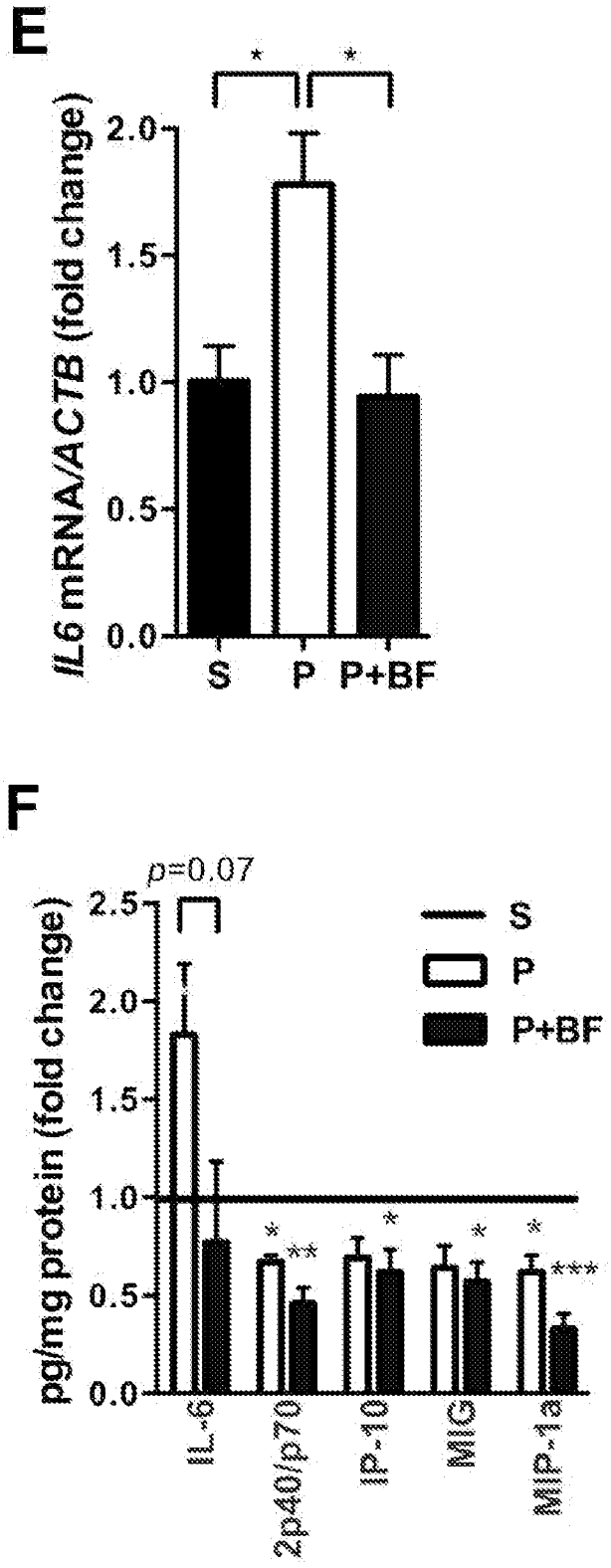


Figure 7

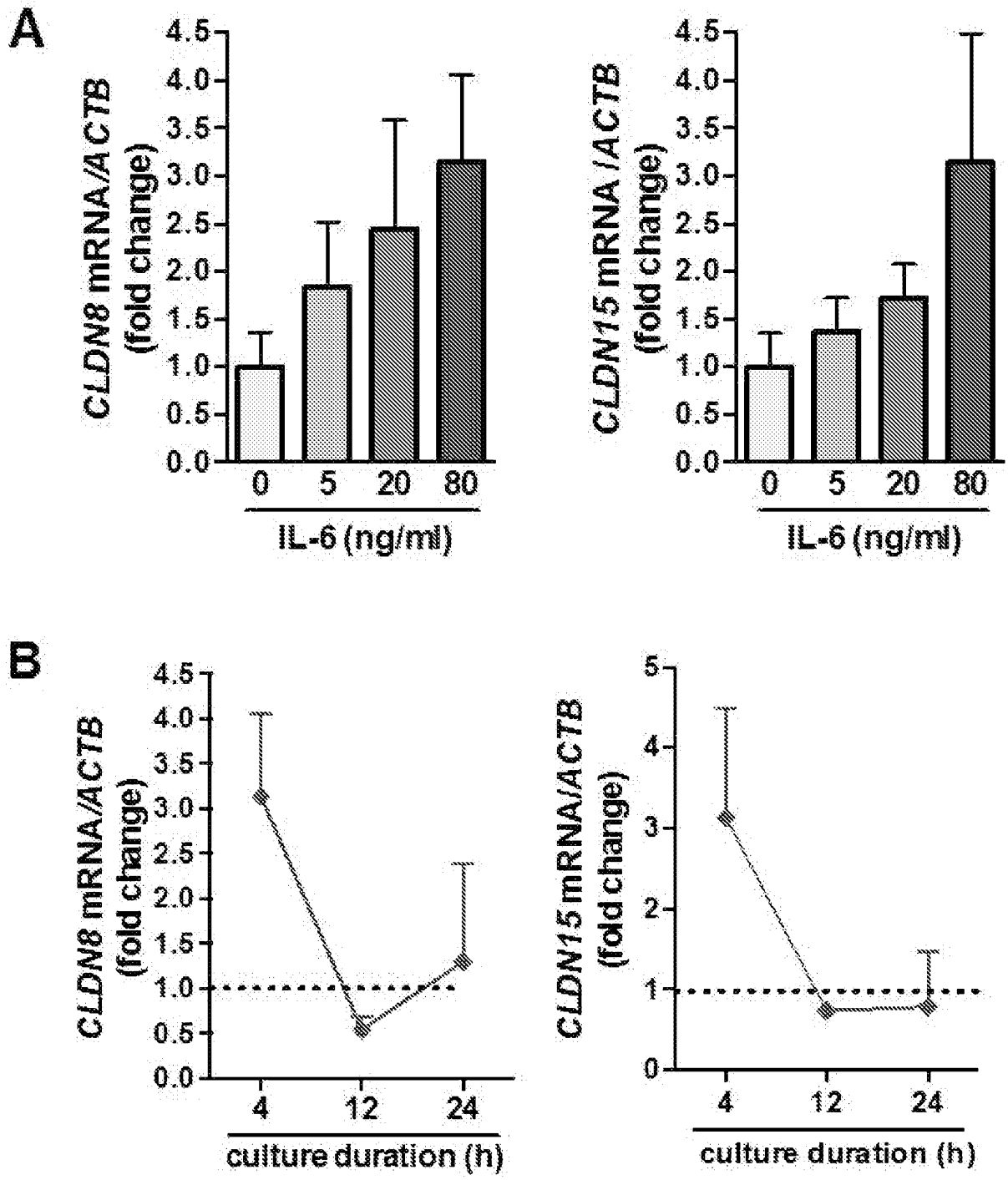

A
CLDN8 mRNA/ACTB (fold change)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
20
80
IL-6 (ng/ml)
CLDN15 mRNA /ACTB (fold change)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
20
80
IL-6 (ng/ml)
B
CLDN8 mRNA/ACTB (fold change)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
12
24
culture duration (h)
CLDN15 mRNA/ACTB (fold change)
5
4
3
2
1
0
4
12
24
culture duration (h)

Figure 7 (continued)

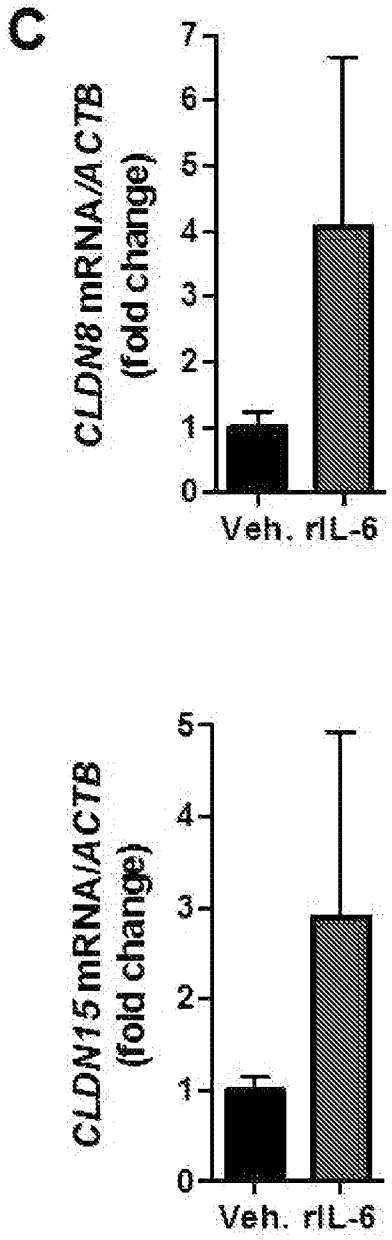


Figure 8

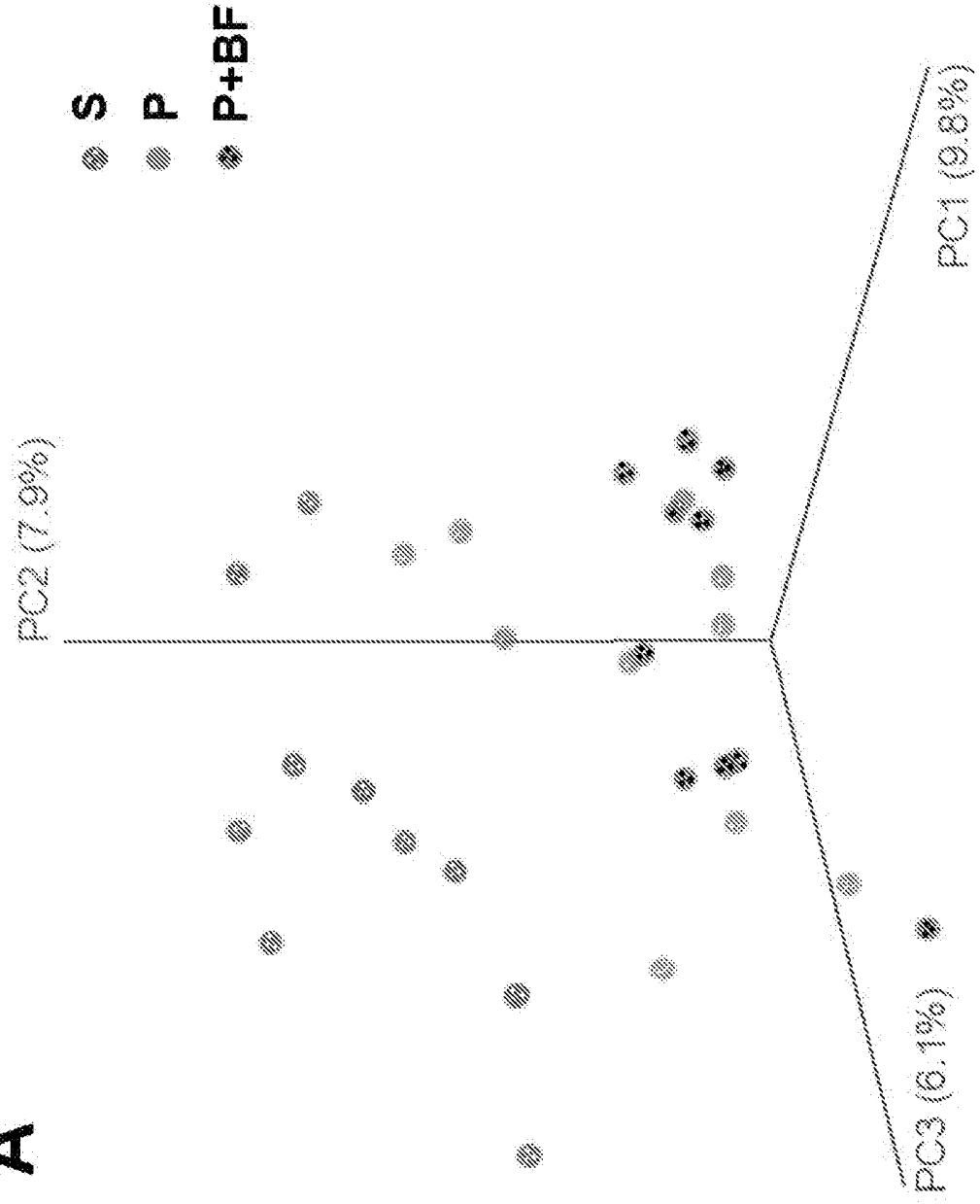


Figure 8 (continued)

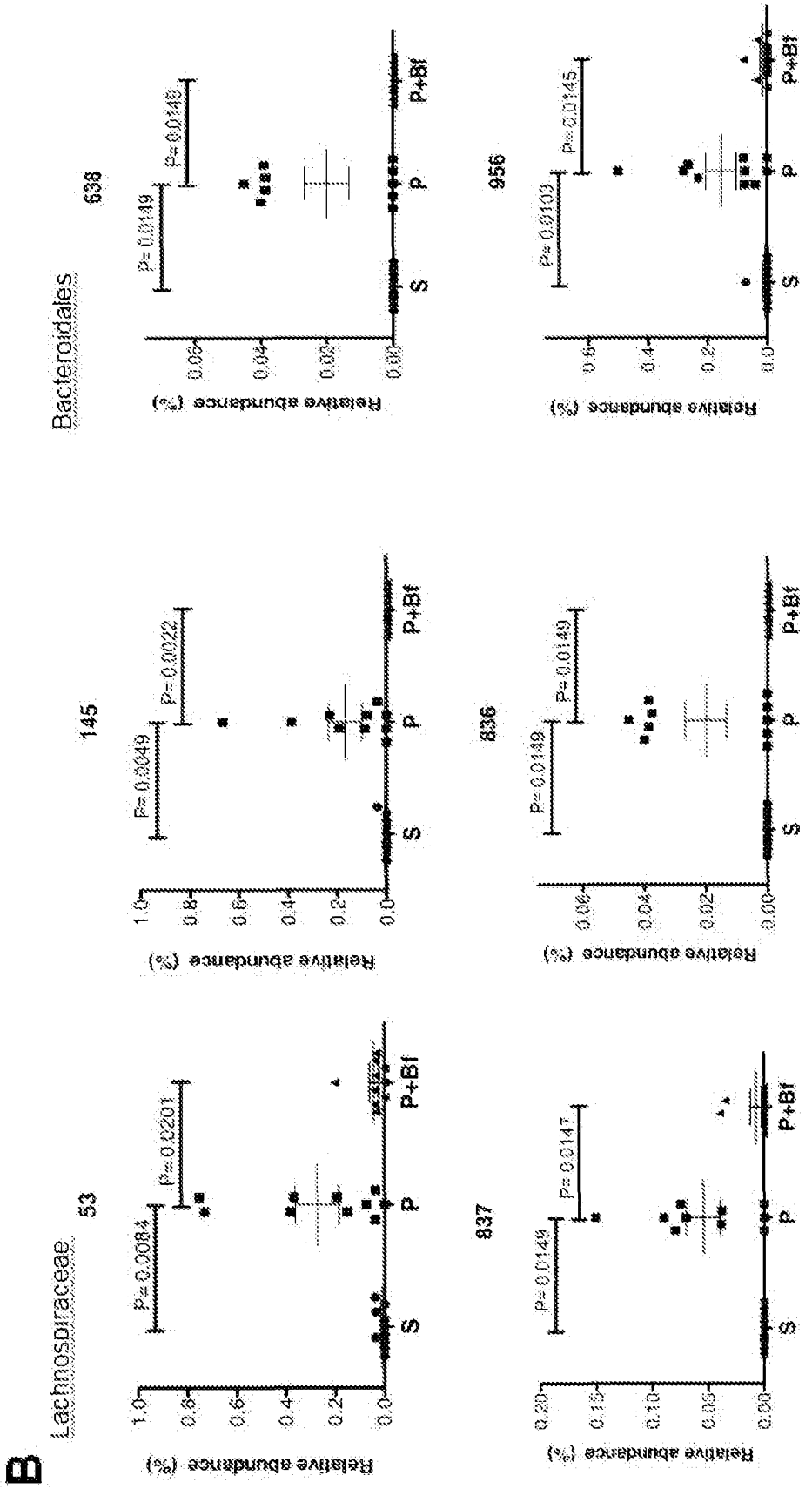


Figure 8 (continued)

C

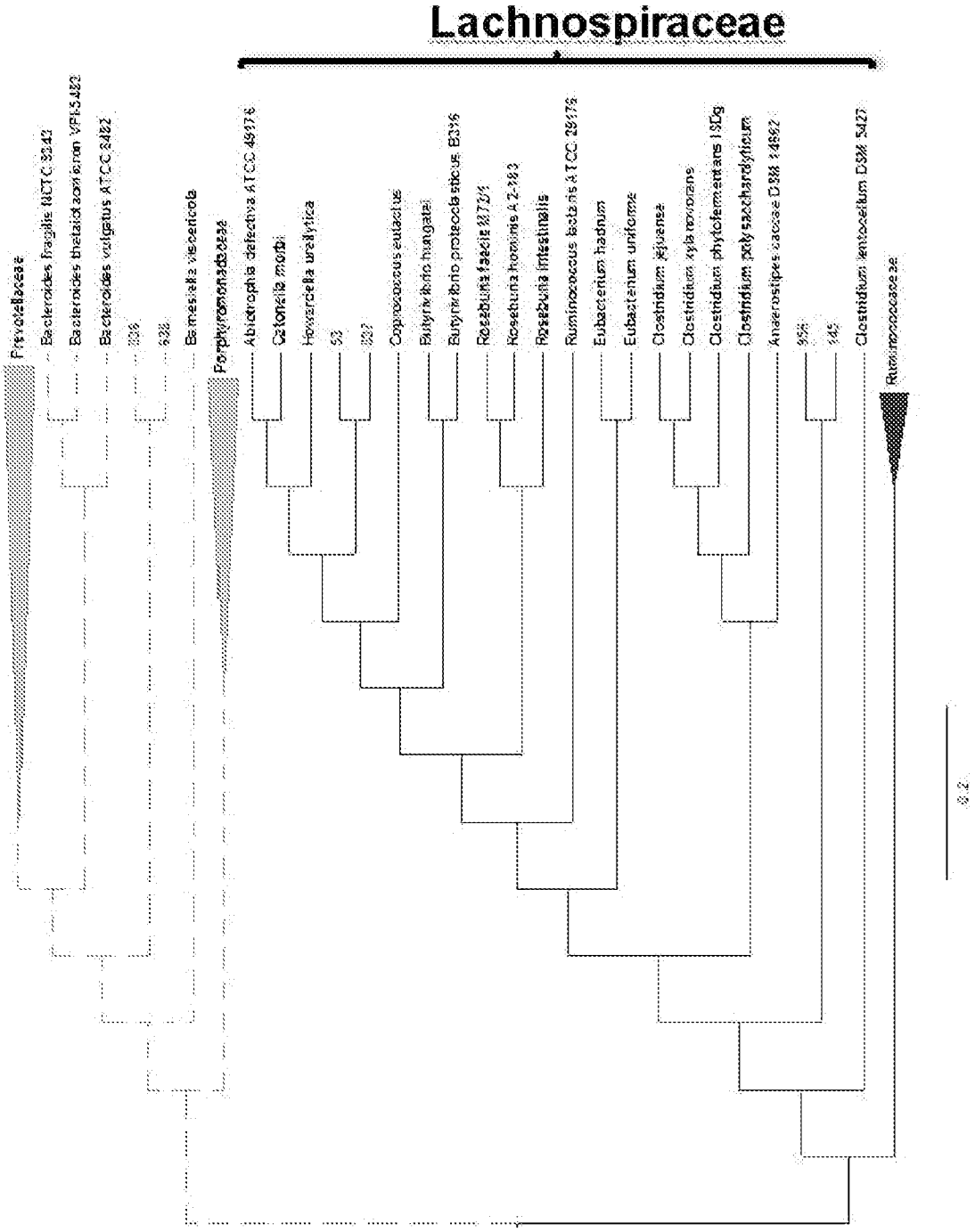


Figure 9

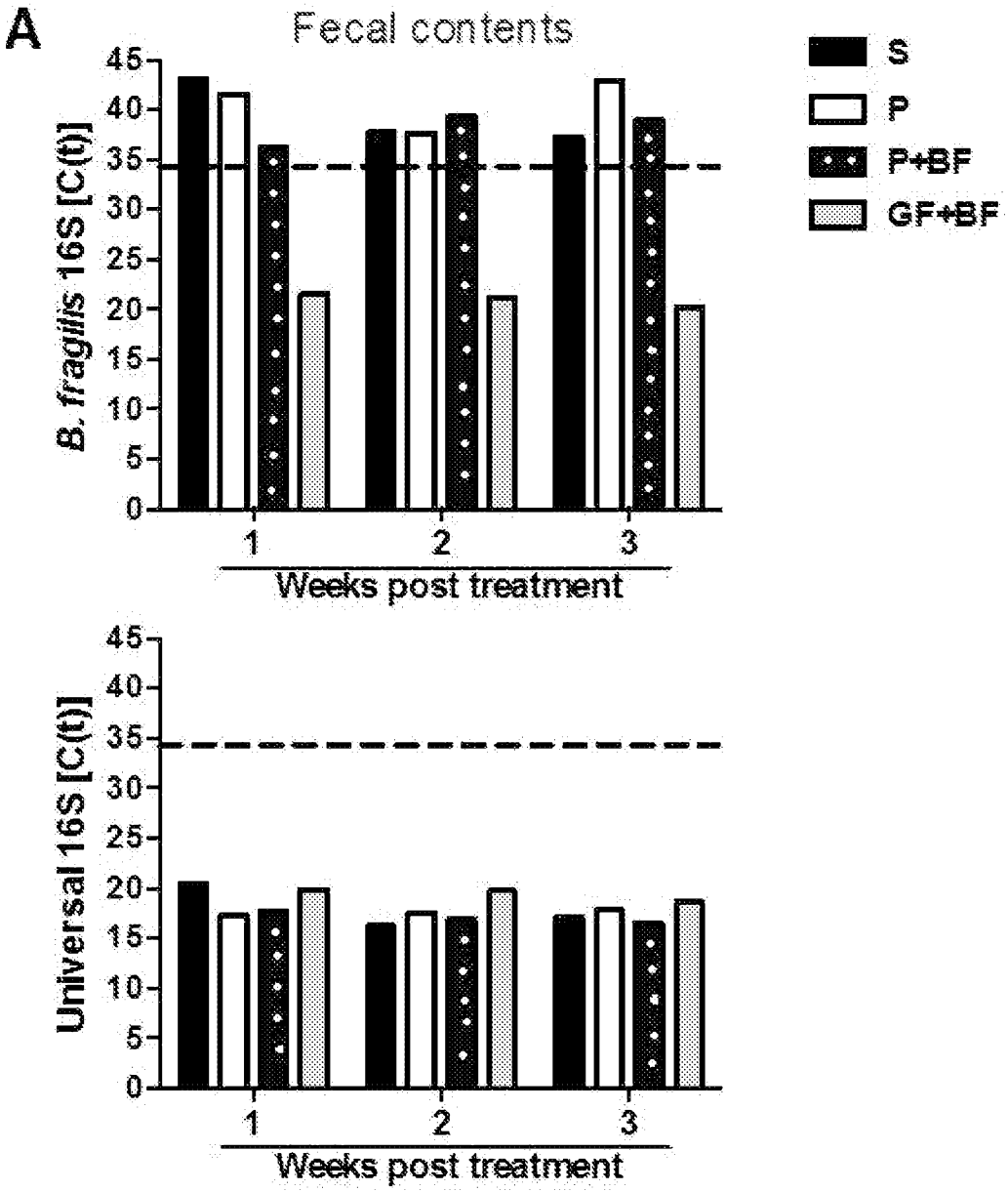

A
Fecal contents
S
P
P+BF
GF+BF
B. fragilis 16S [C(t)]
Universal 16S [C(t)]
Weeks post treatment
Weeks post treatment

Figure 9 (continued)

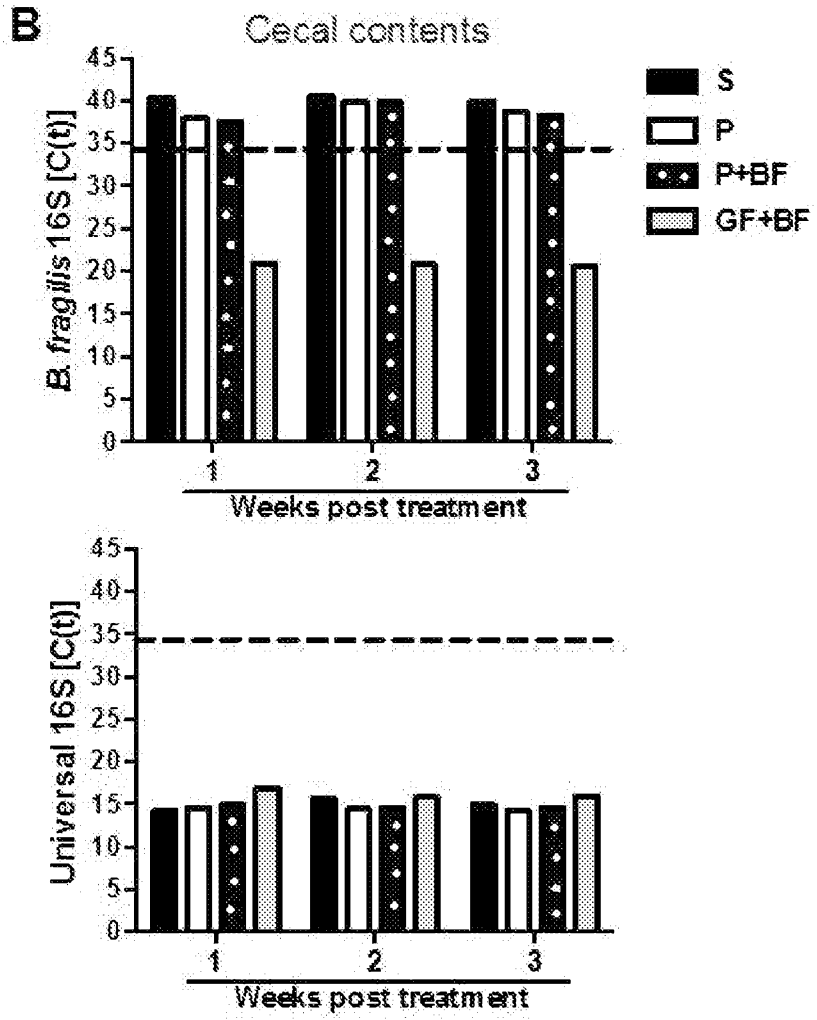


Figure 10

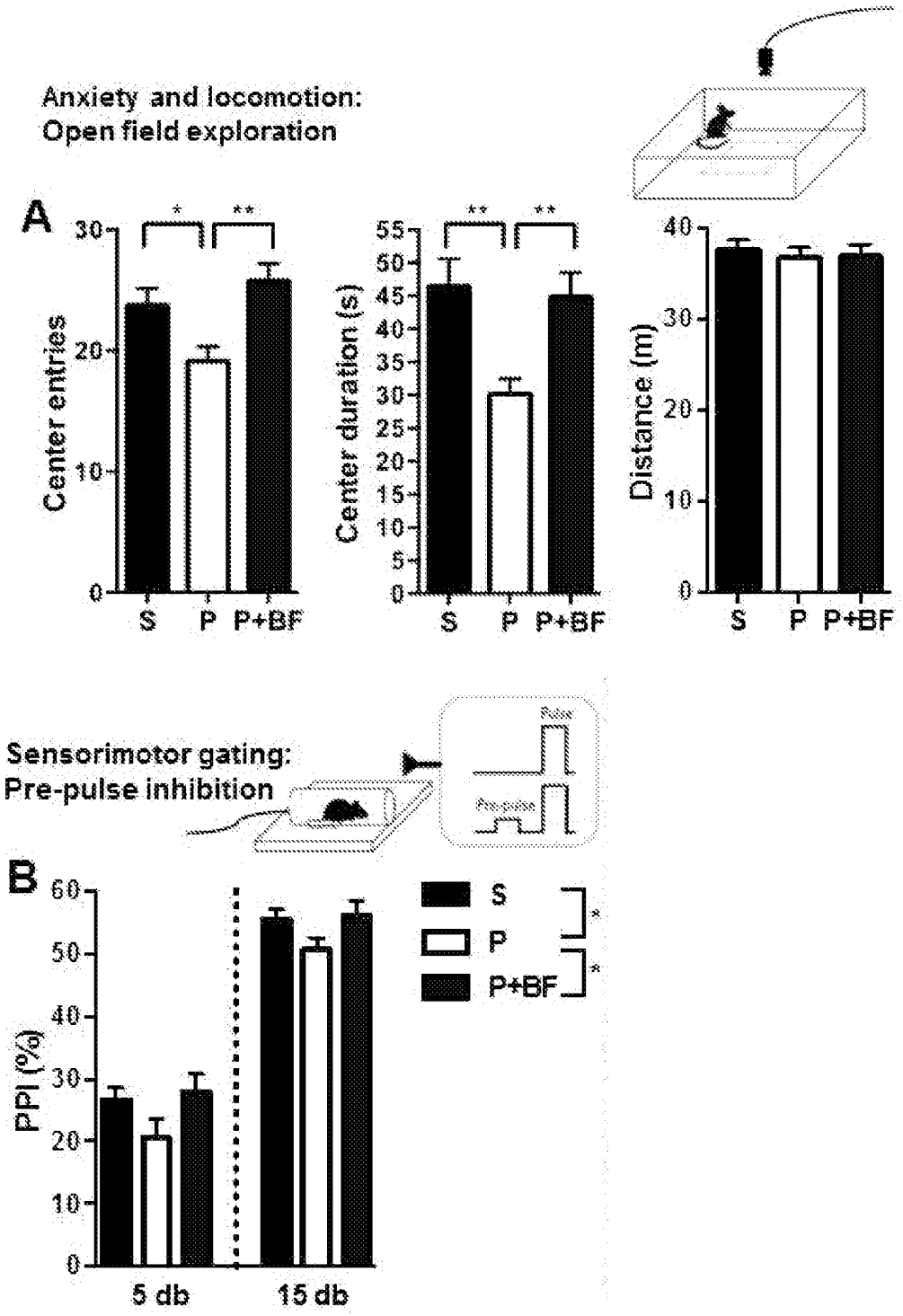

Anxiety and locomotion:
Open field exploration
A
Center entries
Center duration (s)
Distance (m)
S
P
P+BF
Sensorimotor gating:
Pre-pulse inhibition
B
PPI (%)
5 db
15 db
S
P
P+BF

Figure 10 (continued)

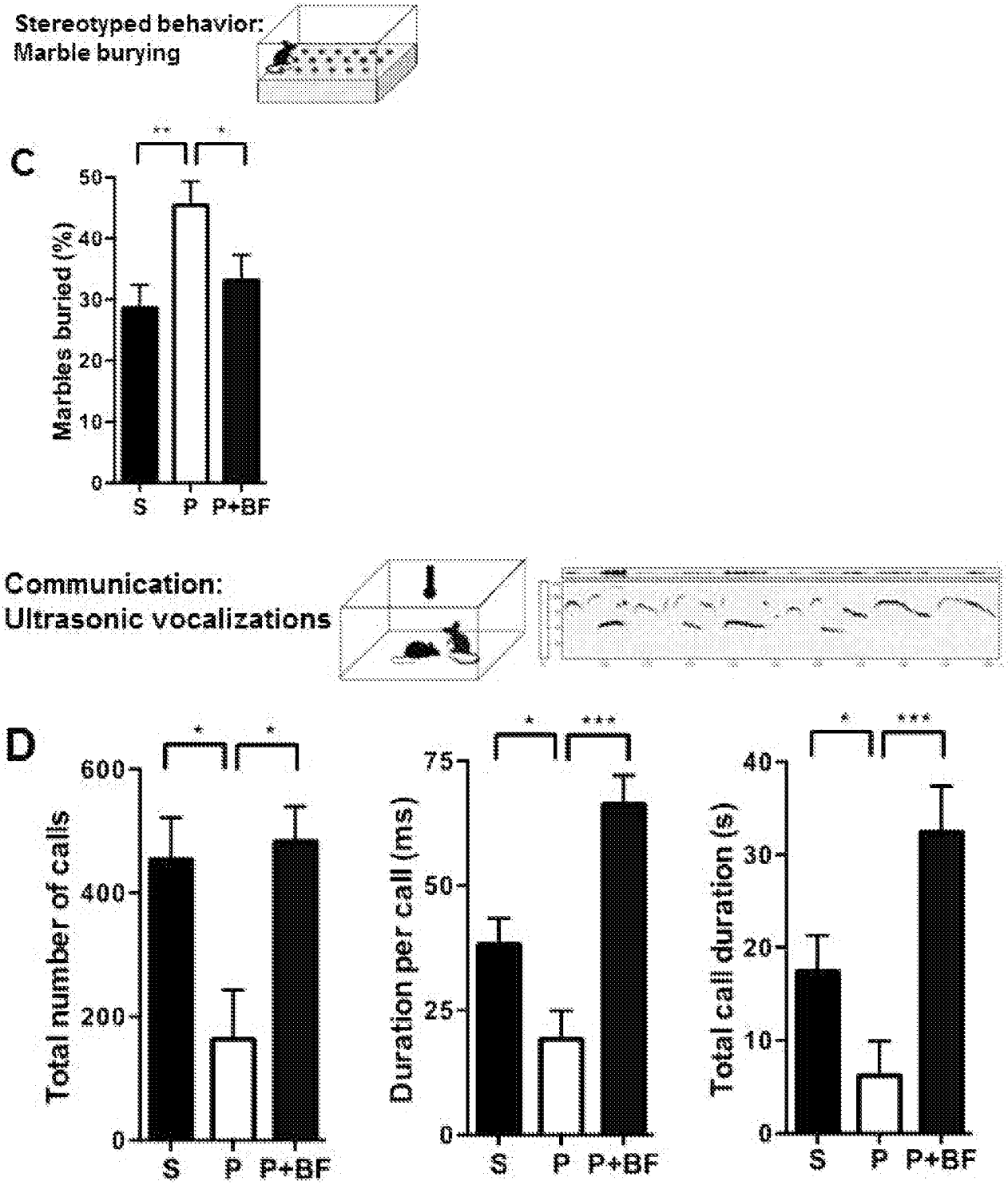


Figure 10 (continued)

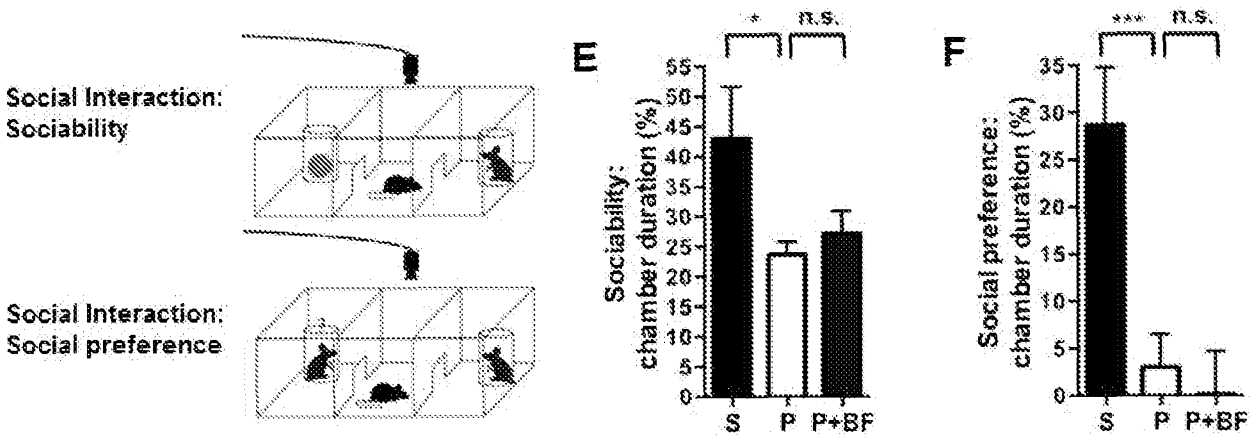


Figure 11

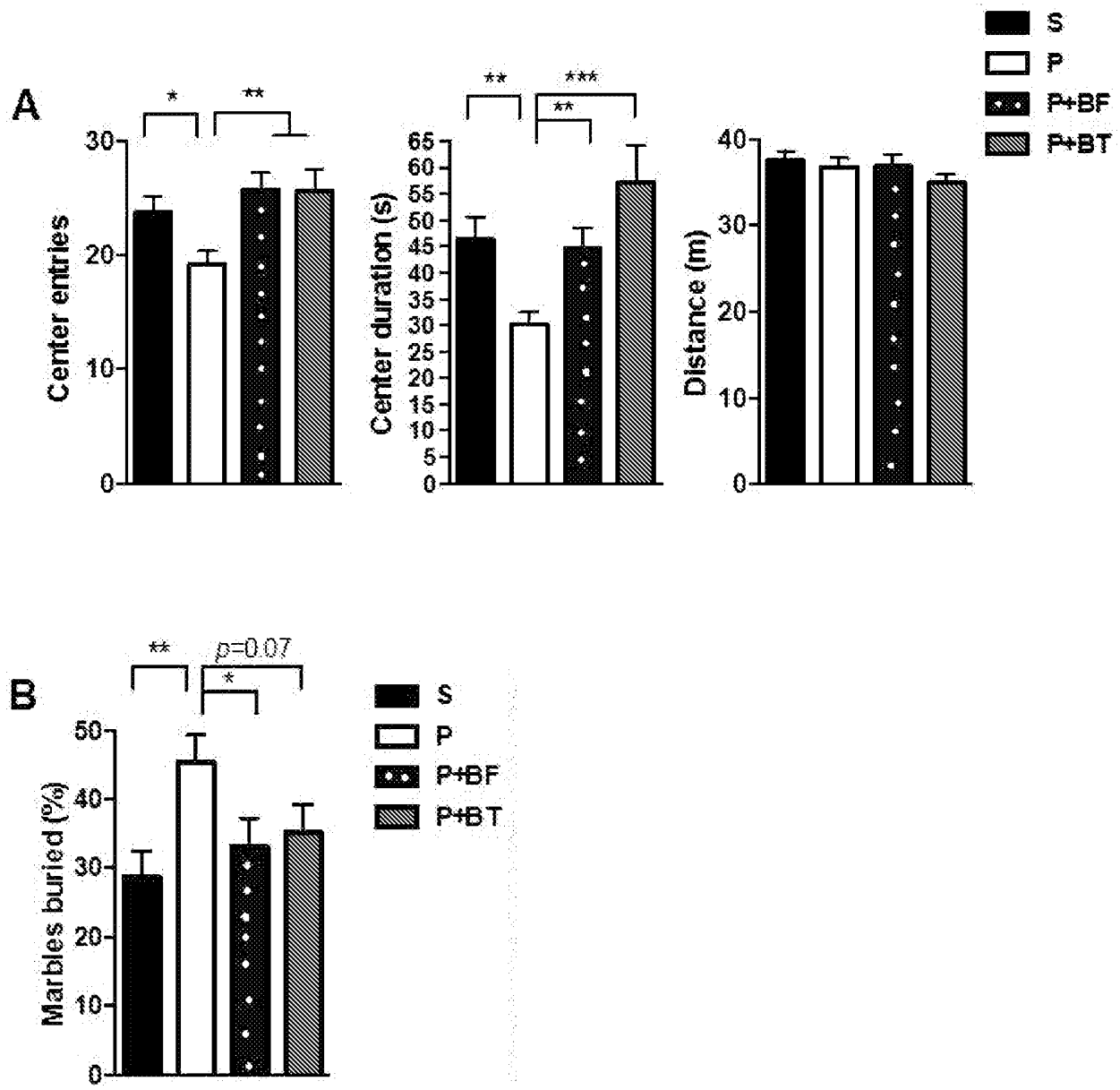

A
Center entries
Center duration (s)
Distance (m)
S
P
P+BF
P+BT
*
**
***
B
Marbles buried (%)
p=0.07

Figure 11 (continued)

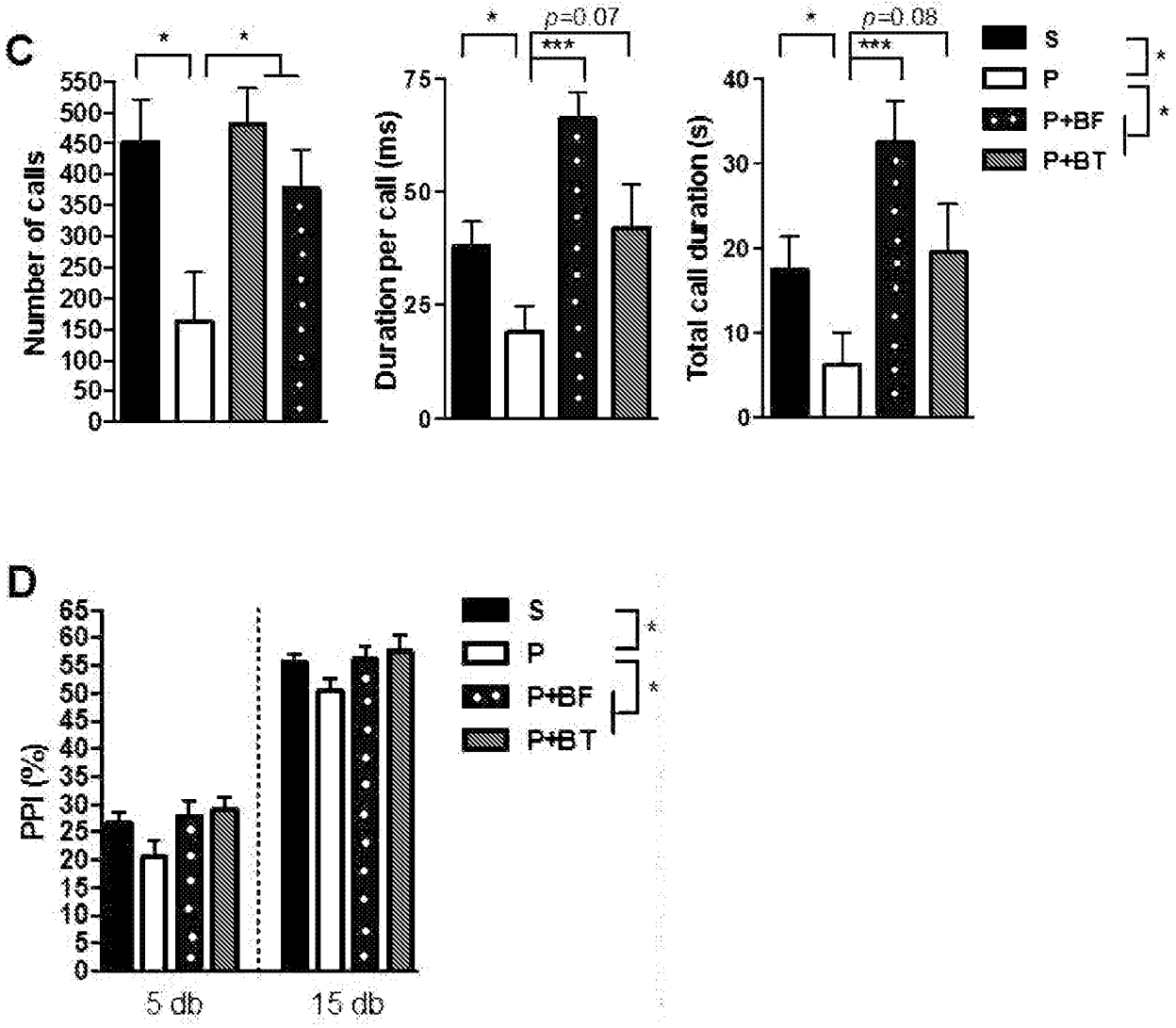


Figure 12A

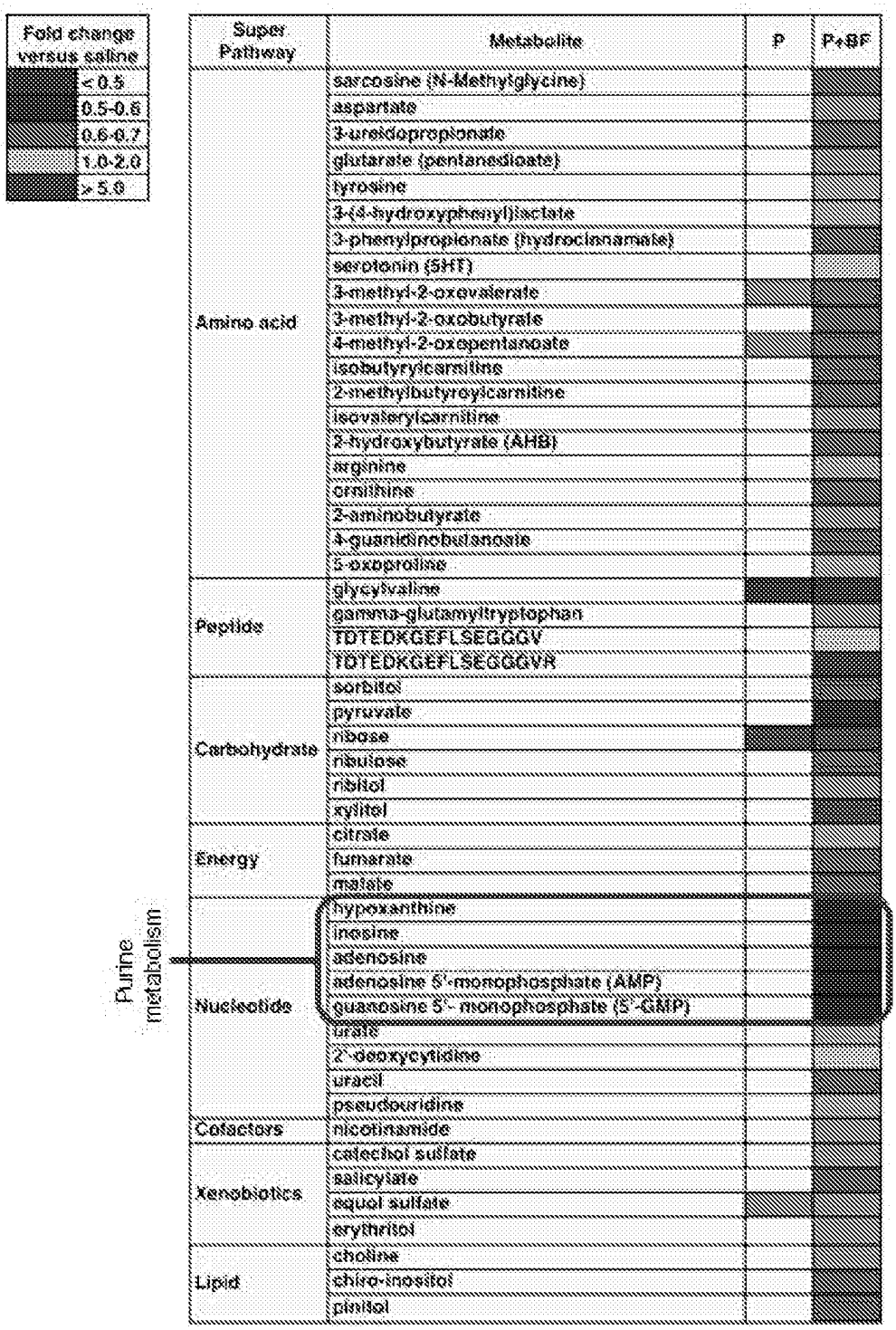

Fold change versus saline
< 0.5
0.5-0.6
0.6-0.7
1.0-2.0
> 5.0
Super Pathway
Metabolite
P
P+BF
Amino acid
sarcosine (N-Methylglycine)
aspartate
3-ureidopropionate
glutarate (pentanedioate)
tyrosine
3-(4-hydroxyphenyl)lactate
3-phenylpropionate (hydrocinnamate)
serotonin (5HT)
3-methyl-2-oxovalerate
3-methyl-2-oxobutyrate
4-methyl-2-oxopentanoate
isobutyrylcarnitine
2-methylbutyrylcarnitine
isovalerylcarnitine
2-hydroxybutyrate (AHB)
arginine
ornithine
2-aminobutyrate
4-guanidinobutanoate
5-oxoproline
Peptide
glycylvaline
gamma-glutamyltryptophan
TDTEDKGEFLSEGGGV
TDTEDKGEFLSEGGGVR
Carbohydrate
sorbitol
pyruvate
ribose
ribulose
ribitol
xylitol
Energy
citrate
fumarate
malate
Purine metabolism
Nucleotide
hypoxanthine
inosine
adenosine
adenosine 5'-monophosphate (AMP)
guanosine 5'- monophosphate (5'-GMP)
urate
2'-deoxycytidine
uracil
pseudouridine
Cofactors
nicotinamide
Xenobiotics
catechol sulfate
salicylate
equol sulfate
erythritol
Lipid
choline
chiro-inositol
pinitol

Figure 12B

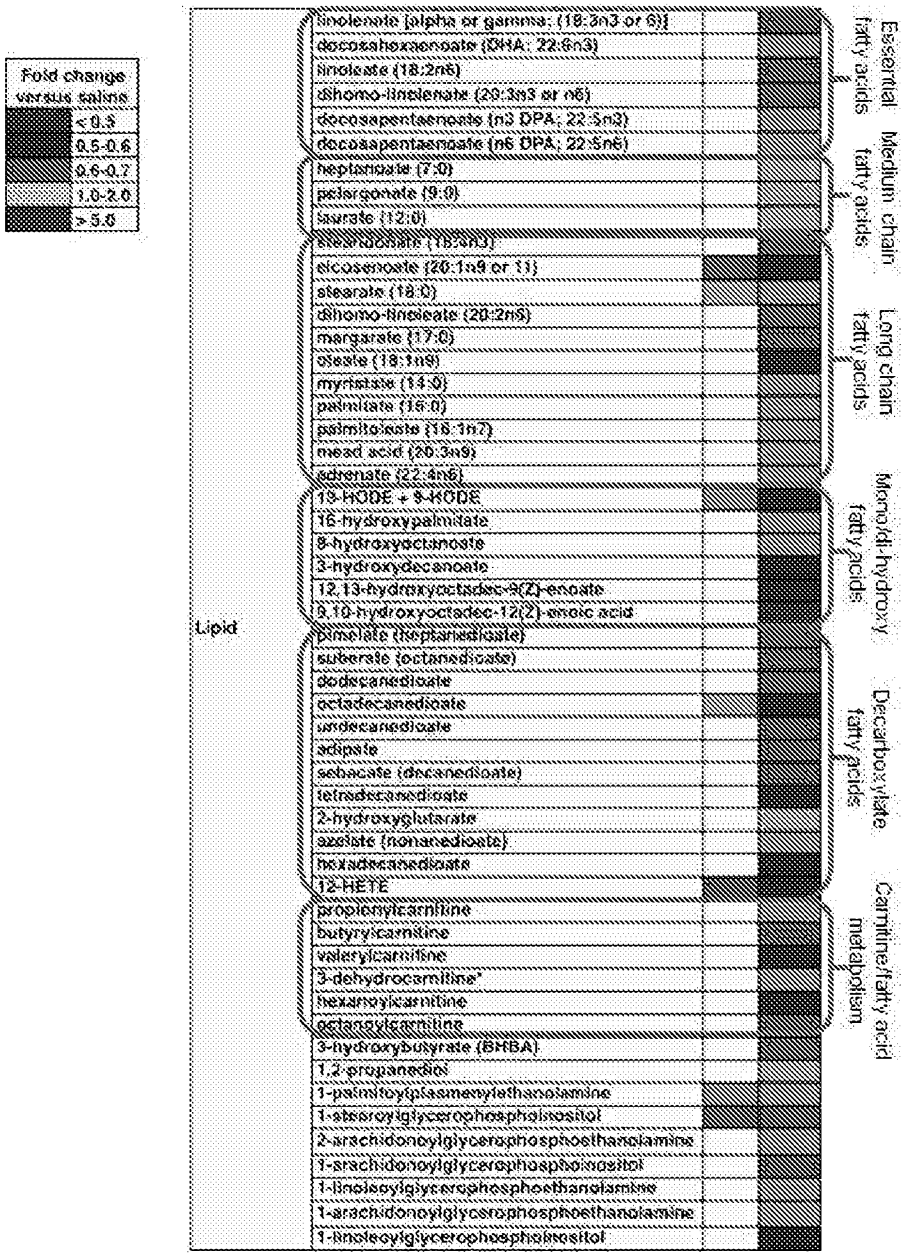

Fold change versus saline
< 0.5
0.5-0.6
0.6-0.7
1.0-2.0
> 5.0
Lipid
linolenate [alpha or gamma; (18:3n3 or 6)]
docosahexaenoate (DHA; 22:6n3)
linoleate (18:2n6)
dihomo-linolenate (20:3n3 or n6)
docosapentaenoate (n3 DPA; 22:5n3)
docosapentaenoate (n6 DPA; 22:5n6)
heptanoate (7:0)
pelargonate (9:0)
laurate (12:0)
eicosenoate (20:1n9 or 11)
stearate (18:0)
dihomo-linoleate (20:2n6)
margarate (17:0)
oleate (18:1n9)
myristate (14:0)
palmitate (16:0)
palmitoleate (16:1n7)
mead acid (20:3n9)
adrenate (22:4n6)
13-HODE + 9-HODE
16-hydroxypalmitate
8-hydroxyoctanoate
3-hydroxydecanoate
12,13-hydroxyoctadec-9(Z)-enoate
9,10-hydroxyoctadec-12(Z)-enoic acid
pimelate (heptanedioate)
suberate (octanedioate)
dodecanedioate
octadecanedioate
undecanedioate
adipate
sebacate (decanedioate)
tetradecanedioate
2-hydroxyglutarate
azelate (nonanedioate)
hexadecanedioate
12-HETE
propionylcarnitine
butyrylcarnitine
valerylcarnitine
3-dehydrocarnitine*
hexanoylcarnitine
octanoylcarnitine
3-hydroxybutyrate (BHBA)
1,2-propanediol
1-palmitoylplasmenylethanolamine
1-stearoylglycerophosphoinositol
2-arachidonoylglycerophosphoethanolamine
1-arachidonoylglycerophosphoinositol
1-linoleoylglycerophosphoethanolamine
1-arachidonoylglycerophosphoethanolamine
1-linoleoylglycerophosphoinositol
Essential fatty acids
Medium chain fatty acids
Long chain fatty acids
Mono/di-hydroxy fatty acids
Decarboxylate fatty acids
Carnitine/fatty acid metabolism

Figure 13

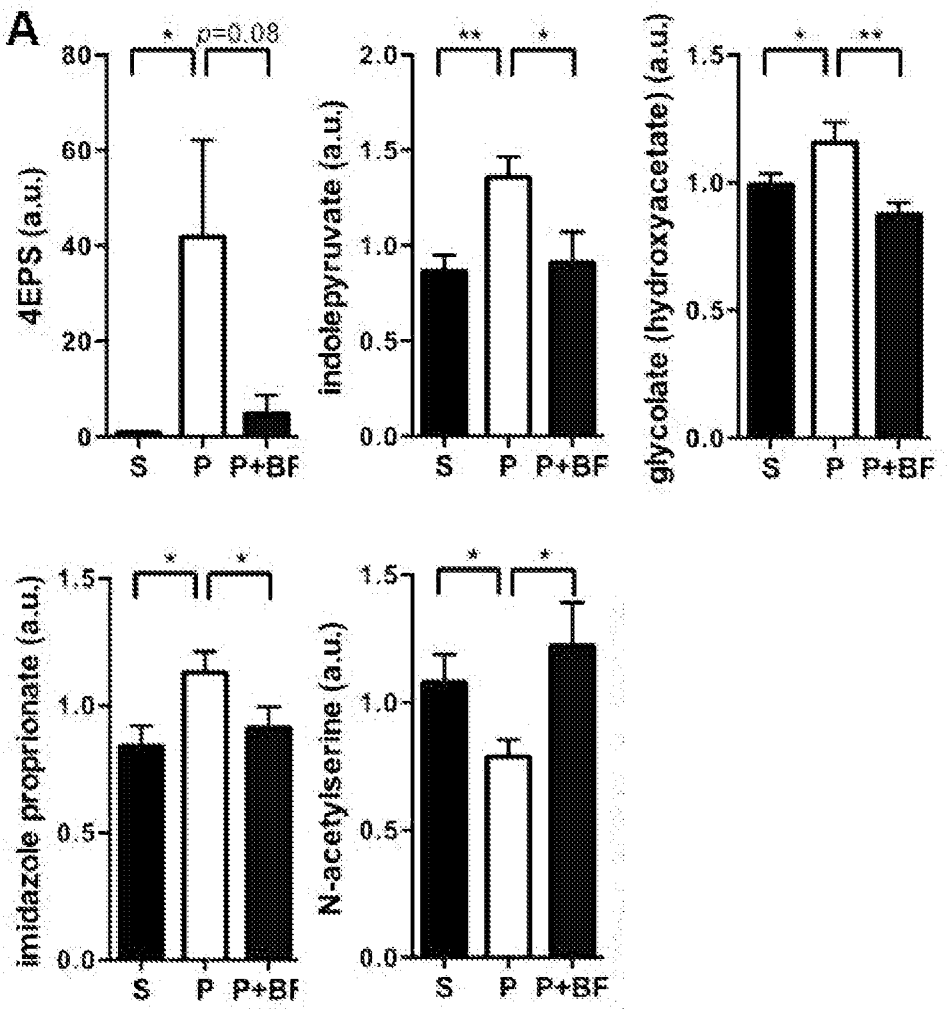

A
4EPS (a.u.)
indolepyruvate (a.u.)
glycolate (hydroxyacetate) (a.u.)
imidazole proprionate (a.u.)
N-acetylserine (a.u.)
S
P
P+BF
p=0.08

Figure 13 (continued)

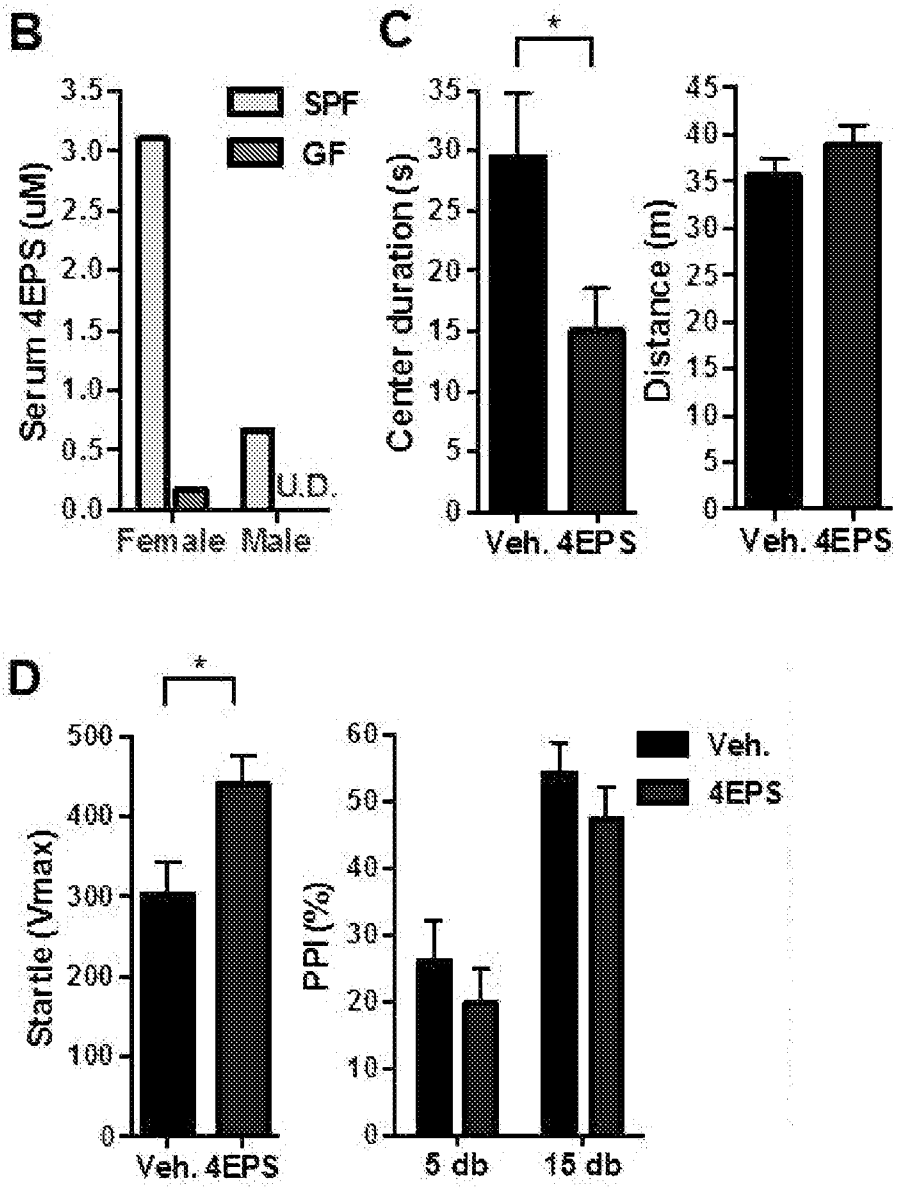


Figure 14A

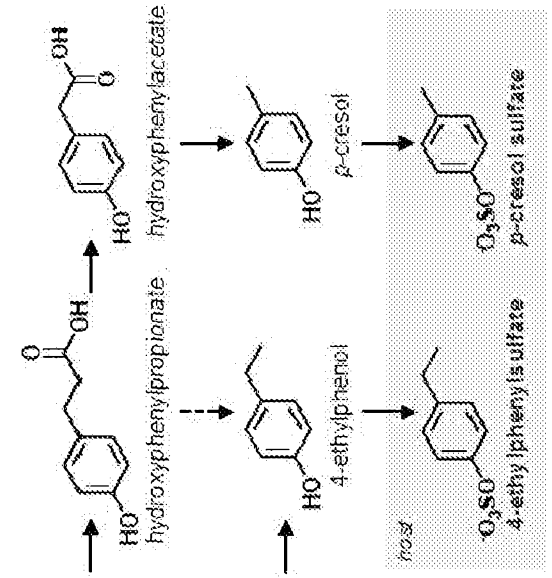


Figure 14B

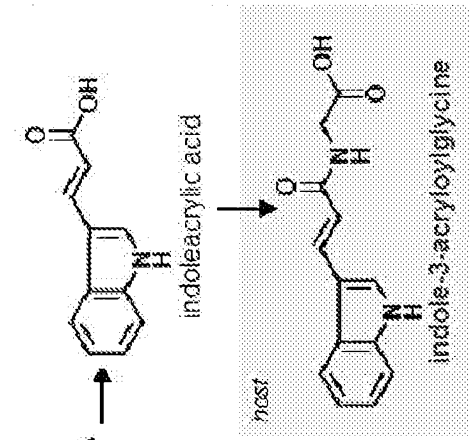


Figure 15A

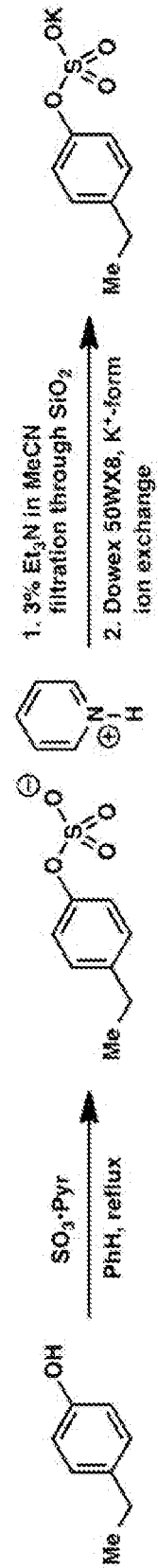


Figure 15B

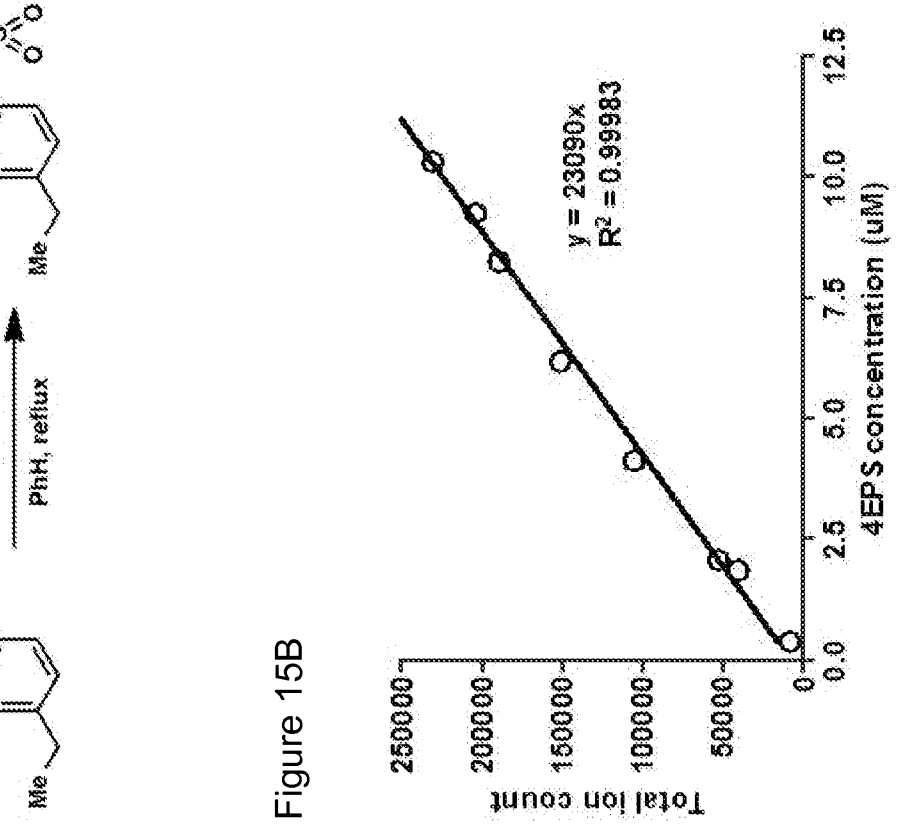


Figure 15C

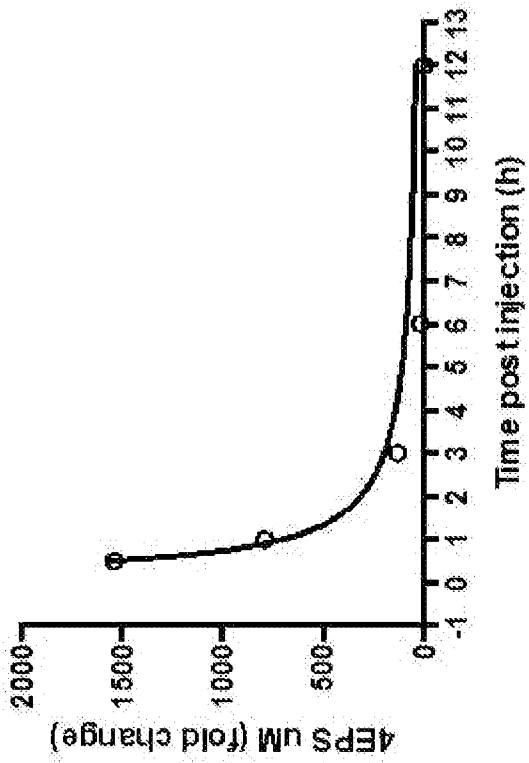


Figure 15D

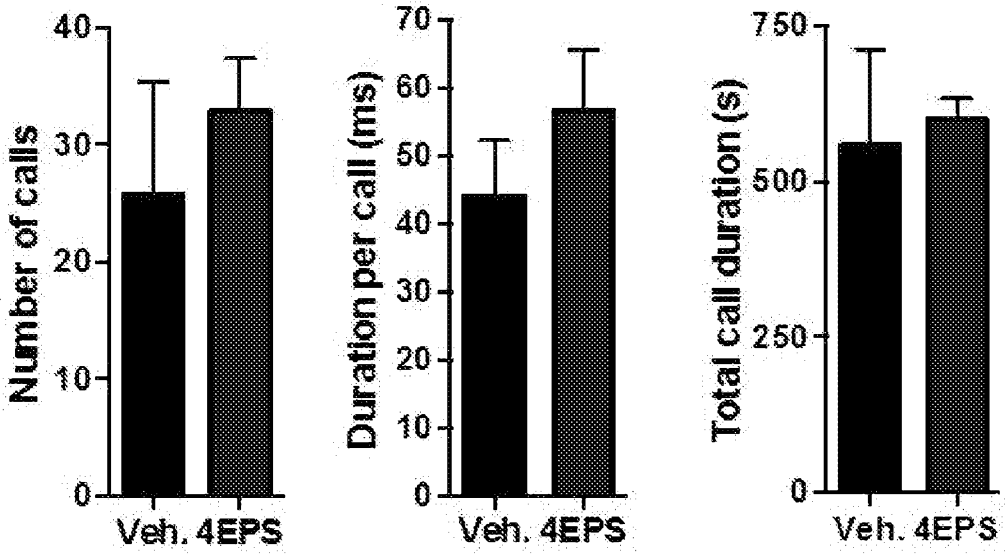


Figure 15E