USE OF BACTERIA
IN CHILDREN DEVELOPMENT ASSESSMENT AND TREATMENT

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/039,034, filed June 15, 2020, and U.S. Provisional Patent Application No. 63/121,198, filed, December 3, 2020, the contents of all of the above are hereby incorporated by reference in the entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Autism spectrum disorder (ASD) is a complex group of developmental disorders characterized by impaired social interactions and communication together with repetitive behaviors. The purpose of this study is to determine bacterial biomarkers for individuals with autism, as well as to identify probiotic/therapeutic bacteria for autism. The gut bacterial profile is different between autism children and typically developing children, and the profile also evolves as children grow and develop. Gut microbiota is regarded as an important factor in the development of ASD as well as indicator of growth and development age of children. Currently there are no effective methods for diagnosing or treating autism and particularly no existing model using microbial markers to predict risk of autism in children, nor any model using microbial markers for assessing developmental age of children. This invention provide a new method for predicting risk of autism in children, new method for improve behavioral symptom in autism patients by microbial transfer and/or supplementation, and new method for assessing children's developmental age based on their gut microbial profile.

BRIEF SUMMARY

[0003] The disclosure relates to novel methods and compositions useful for treating the symptoms of autism spectrum disorder (ASD). In particular, the present inventor discovered that certain microorganism species, especially certain bacteria, are at an altered level, in the gastrointestinal (GI) tract of children at risk for ASD or suffering from ASD. Health benefits such as improving behavioral symptoms and alleviating detrimental effects can be achieved by modulating the level of pertinent microorganisms in patients' gut, for example, by fecal

microbiota transplantation (FMT) treatment or oral administration of beneficial bacterial species or by suppressing the level of harmful bacterial species. These findings also provide new methods indicating the presence or risk of ASD in a child. Thus, in the first example, the present disclosure provides a novel method for treating ASD, including alleviating ASD symptoms, by increasing the level of one or more bacterial species named in Table 1 in the gastrointestinal tract of a child afflicted by ASD.

[0003a] In a first aspect, the present disclosure relates to a method for assessing risk for autism spectrum disorder (ASD) in a human child, comprising: (1) determining, in a stool sample which is to be obtained from the child, the level or relative abundance of one or more of the bacterial species set forth in Table 3; (2) determining the level or relative abundance of the same bacterial species in a stool sample from a reference cohort comprising normal and ASD children; (3) generating decision trees by random forest model using data obtained from step (2) and running the level or relative abundance of one or more of the bacterial species from step (1) down the decision trees to generate a risk score; and (4) determining the child with a risk score greater than 0.5 as having an increased risk for ASD and determining the child with a risk score no greater than 0.5 as having no increased risk for ASD.

[0003b] In a second aspect, the present disclosure relates to a method for treating symptoms of autism spectrum disorder (ASD) in a human child, comprising introducing into the child's gastrointestinal tract an effective amount of one or more of the bacterial species of *Faecalibacterium prausnitzi*, *Roseburia inulinivorans*, *Eubacterium hallii*, *Dorea longicatena*, or *Eubacterium siraeum.*

[0003c] In a third aspect, the present disclosure relates to a method for treating symptoms of autism spectrum disorder (ASD) in a human child, comprising reducing the level or relative abundance of one or more of the bacterial species of *Clostridium nexile*, *Dialister invisus*, *Clostridium bolteae*, *Clostridium symbiosum*, *Eubacterium limosum*, *Clostridiales bacterium_1_7_47FAA*, *Clostridium ramosum*, *Anaerotruncus colihominis*, *Clostridium citroniae*, or *Alistipes indistinctus* in the child's gastrointestinal tract.

[0003d] In a fourth aspect, the present disclosure relates to a kit for treating symptoms of ASD, comprising: a first container containing a first composition comprising (i) an effective amount of one of the bacterial species set forth in Table 1, or (ii) an effective amount of an anti-bacterial agent that suppresses growth of one of the bacterial species set forth in Table 2, and a second container containing a second composition comprising (i) an effective amount of another one of the bacterial species set forth in Table 1, or (ii) an effective amount of an

anti-bacterial agent that suppresses growth of another one of the bacterial species set forth in Table 2.

[0003e] In a fifth aspect, the present disclosure relates to a method for determining risk for autism spectrum disorder (ASD) in a human child, comprising: (1) determining, in a stool sample from the child, the relative abundance of any one of the bacterial species set forth in Table 1 or 2; and (2) detecting the relative abundance from step (1) being no lower than the cutoff value in Table 1 or a standard control value or being lower than the cutoff value or a standard control value in Table 2 and determining the child as not having increased risk for ASD; or detecting the relative abundance from step (1) being lower than the cutoff value in Table 1 or a standard control value or being no lower than the cutoff value in Table 2 or a standard control value and determining the child as having an increased risk for ASD.

[0003f] In a sixth aspect, the present disclosure relates to a method for assessing risk for autism spectrum disorder (ASD) in two human children, comprising: (1) determining, in a stool sample from each of the two children, the relative abundance of any one of the bacterial species set forth in Table 1 or 2; (2) determining the relative abundance of a bacterial species set forth in Table 1 from step (1) being higher in the stool sample from the first child or the relative abundance of a bacterial species set forth in Table 2 from step (1) being lower in the stool sample from the first child; and (3) determining the second child as having a higher risk for ASD than the first child.

[0003g] In a seventh aspect, the present disclosure relates to a method for determining risk for autism spectrum disorder (ASD) in a human child, comprising: (1) obtaining in a stool sample from the child a value of (a) the relative abundance of *Alistipes indistinctus* (Ai) or *Anaerotruncus colihominis* (Ac), or (b) the combined score of levels of three bacterial species Ai, Ac, and *Eubacterium hallii* (Eh), which is calculated by by $I1 + \beta1*Ai + \beta2*Eh + \beta3*Ac$; and (2) detecting the value to be higher than a standard control value and determining the individual as having increased risk of ASD.

[0003h] In an eighth aspect, the present disclosure relates to a method for determining risk for autism spectrum disorder (ASD) in a human child, comprising: (1) obtaining in a stool sample from the child a value of the relative abundance of *Eubacterium hallii* (Eh); and (2) detecting the value to be lower than a standard control value and determining the individual as having increased risk of ASD.

[0003i] In a ninth aspect, the present disclosure relates to a kit for assessing risk for autism spectrum disorder (ASD), comprising reagents for detecting one or more of the bacterial species set forth in Table 1, 2, or 3.

[0003j] In a tenth aspect, the present disclosure relates to a method for determining developmental age of a child, comprising the steps of: (a) quantitatively determining the relative abundance of one or more bacterial species selected from Table 8 or 9 in a stool sample taken from the child; (b) quantitatively determining the relative abundance of the one or more bacterial species in a stool sample taken from a reference cohort consisting of typically developing children; (c) generating decision trees by random forest model using data obtained from step (b); and (d) running the relative abundances obtained from step (a) down the decision trees from step (b) to generate a developmental age for the child.

[0003k] In an eleventh aspect, the present disclosure relates to a kit for determining developmental age of a child, comprising: a first container containing a first reagent for detecting a first bacterial species set forth in Table 8 or 9, and a second container containing a second reagent for detecting a second bacterial species set forth in Table 8 or 9.

[0003l] In a twelfth aspect, the present disclosure relates to a method for promoting growth and development of a child, comprising administering to the child an effective amount of one or more bacterial species selected from Table 8.

[0003m] In a thirteenth aspect, the present disclosure relates to a kit for promoting growth and development of a child, comprising: a first container containing a first composition comprising (i) an effective amount of one of the bacterial species set forth in Table 8, and a second container containing a second composition comprising (i) an effective amount of another one of the bacterial species set forth in Table 8.

[0004] In some examples, the introducing step comprises oral administration to the subject a composition comprising an effective amount of the one or more of the bacterial species. In some examples, the introducing step comprises delivery to the small intestine, ileum, or large intestine of the subject a composition comprising an effective amount of the one or more of the bacterial species. In some examples, the introducing step comprises fecal microbiota transplantation (FMT). In some examples, the FMT comprises administration to the child a composition comprising processed donor fecal material. In some examples, the composition is orally administered; or the composition is directly deposited to the child's gastrointestinal tract. In some examples, the level or relative abundance of the one or more of the bacterial species is determined in a first stool sample obtained from the child prior to the introducing

step and in a second stool sample obtained from the child after the introducing step. In some examples, the level of the one or more of the bacterial species is determined by polymerase chain reaction (PCR), especially quantitative PCR.

[0005] In a second example, the present disclosure provides a method for treating ASD, including alleviating ASD symptoms, by reducing the level of one or more bacterial species in Table 2 in the gastrointestinal tract of a child afflicted by ASD.

[0006] In some examples, the reducing step comprises FMT. In some examples, the reducing step comprises treating the subject with an anti-bacterial agent. In some examples, a composition comprising processed donor fecal material is introduced to the gastrointestinal tract of the subject after the subject is treated with the anti-bacterial agent. For example, the composition is orally administered, or the composition is directly deposited to the gastrointestinal tract of the child. In some examples, the level or relative abundance of the one or more of the bacterial species is determined in a first stool sample obtained from the child prior to the reducing step and in a second stool sample obtained from the child after the reducing step. In some examples, the level of the one or more bacterial species is determined by PCR, especially by quantitative PCR.

[0007] In a related example, a kit is provided for treating the symptoms of ASD. The kit comprises: a first container containing a first composition comprising (i) an effective amount of a first one of the bacterial species set forth in Table 1, or (ii) an effective amount of an anti-bacterial agent that suppresses growth of a first one of the bacterial species set forth in Table 2, and a second container containing a second composition comprising (i) an effective amount of a second one of the bacterial species set forth in Table 1, or (ii) an effective amount of an anti-bacterial agent that suppresses growth of a second one of the bacterial species set forth in Table 2.

[0008] In some examples, the first composition comprises processed donor fecal material for FMT, for example, the material has been processed and formulated for oral administration, such as dried, frozen or lyophilized, and placed in a capsule suitable for oral ingestion. In some examples, the second composition is formulated for oral administration. In some examples, both the first and second compositions are formulated for oral administration. In some cases, the kit may include two or more compositions each comprising an effective amount of at least one, possibly two or more, of the bacterial species set forth in Table 1, and/or (ii) an effective amount of an anti-bacterial agent that suppresses growth of at least one, possibly two or more, of the bacterial species set forth in Table 2. The compositions in the kit may each comprise a physiologically acceptable carrier or excipient.

[0009] In a third example, a method is provided for determining risk for autism spectrum disorder (ASD) in a human child. The method comprises these steps: (1) determining, in a stool sample taken from the child, the relative abundance of any one of the bacterial species set forth in Table 1 or 2; and (2) detecting the relative abundance from step (1) being no lower than the cutoff value in Table 1 or a standard control value or being lower than the cutoff value in Table 2 or a standard control value and determining the child as not having increased risk for ASD; or detecting the relative abundance from step (1) being lower than the cutoff value in Table 1 or a standard control value or being no lower than the cutoff value in Table 2 or a standard control value and determining the child as having an increased risk for ASD. In some examples, the relative abundance of the bacterial species in the child's stool sample is determined by PCR, *e.g.*, quantitative PCR.

[0010] In a related example, a method is provided for assessing risk for autism spectrum disorder (ASD) in two human children. The method comprises these steps: (1) determining, in a stool sample from each of the two children, the relative abundance of any one of the bacterial species set forth in Table 1 or 2; and (2) determining the relative abundance of a

bacterial species set forth in Table 1 from step (1) being higher in the stool sample from the first child or the relative abundance of a bacterial species set forth in Table 2 from step (1) being lower in the stool sample from the first child; and (3) determining the second child as having a higher risk for ASD than the first child. In some examples, the relative abundance of the bacterial species in both children's stool samples is determined by PCR, *e.g.*, quantitative PCR.

[0011] Further, a method is provided for determining risk for ASD in a human child including these steps: (1) determining in a stool sample from the child a value of (a) the relative abundance of *Alistipes indistinctus* (Ai) or *Anaerotruncus colihominis* (Ac), or (b) the combined score of levels of three bacterial species Ai, Ac, and *Eubacterium hallii* (Eh), which is calculated by $I1 + \beta1*Ai + \beta2*Eh + \beta3*Ac$; and (2) detecting the value to be higher than a standard control value and determining the individual as having increased risk of ASD.

[0012] Similarly, a method is provided for determining risk of ASD in a human child including these steps: (1) determining in a stool sample from the child relative abundance of *Eubacterium hallii* (Eh); and (2) detecting the relative abundance from step (1) to be lower than a standard control value and determining the individual as having increased risk of ASD.

[0013] In a four example, a method is provided for assessing risk for autism spectrum disorder (ASD) in a human child. The method comprises these steps: (1) determining, in a stool sample from the child, the level or relative abundance of one or more of the bacterial species set forth in Table 3; (2) determining the level or relative abundance of the same bacterial species in a stool sample from a reference cohort comprising normal and ASD children; (3) generating decision trees by random forest model using data obtained from step (2) and running the level or relative abundance of one or more of the bacterial species from step (1) down the decision trees to generate a risk score; and (4) determining the child with a risk score greater than 0.5 as having an increased risk for ASD and determining the child with a risk score no greater than 0.5 as having no increased risk for ASD.

[0014] In some examples, the one or more bacterial species comprise or consist of *Alistipes indistinctus*. In some examples, the one or more bacterial species comprise or consist of *Alistipes indistinctus*, *candidate division TM7 single-cell isolate TM7c*, and *Streptococcus cristatus*. In some examples, the one or more bacterial species comprise or consist of *Alistipes indistinctus*, *candidate division TM7 single-cell isolate TM7c*, *Streptococcus cristatus*, *Eubacterium_limosum*, and *Streptococcus_oligofermentans*.

[0015] In a related example, the present disclosure provides a kit for assessing individuals' risk of developing autism spectrum disorder (ASD). The kit comprises reagents for detecting one or more of the bacterial species set forth in Table 1, 2, or 3. In some examples, the reagents comprise a set of oligonucleotide primers for amplification of a polynucleotide sequence from any one of the bacterial species set forth in Table 1, 2, or 3. In some examples, the amplification is PCR, for example, quantitative PCR.

[0016] In a fifth example, the present disclosure provides a method for determining the growth or developmental age of a child. The method comprises these steps: (a) quantitatively determining the relative abundance of one or more bacterial species selected from Table 8 or 9 in a stool sample taken from the child; (b) quantitatively determining the relative abundance of the one or more bacterial species in a stool sample taken from a reference cohort consisting of typically developing children; (c) generating decision trees by random forest model using data obtained from step (b); and (d) running the relative abundances obtained from step (a) down the decision trees from step (b) to generate a developmental age for the child. In some examples, the one or more bacterial species comprise *Streptococcus gordonii*, *Enterococcus avium*, *Eubacterium_sp_3_1_31*, *Clostridium hathewayi*, and *Corynebacterium durum*. In some examples, the one or more bacterial species comprise *Streptococcus gordonii*, *Enterococcus avium*, *Eubacterium_sp_3_1_31*, and *Clostridium hatheway*. In some examples, the one or more bacterial species comprise *Streptococcus gordonii*, *Enterococcus avium*, and *Eubacterium_sp_3_1_31*. In some examples, the one or more bacterial species comprise *Streptococcus gordonii* and *Enterococcus avium*. In some examples, the one or more bacterial species comprise *Streptococcus gordonii*. In some examples, the child is between about 3 to about 6 years old.

[0017] In a related example, a kit is provided for determining the growth or developmental age of a child. The kit includes a first container containing a first reagent for detecting a first bacterial species set forth in Table 8 or 9 and a second container containing a second reagent for detecting a second and different bacterial species set forth in Table 8 or 9. In some examples, the kit includes three or more containers, each of which containing a reagent for detecting a different bacterial species set forth in Table 8 or 9. In some examples, the kit includes two or more containers, each of which containing a reagent for detecting a different bacterial species selected from the group consisting of (1) *Streptococcus gordonii, Enterococcus avium, Eubacterium_sp_3_1_31, Clostridium hathewayi*, and *Corynebacterium durum*; (2) *Streptococcus gordonii, Enterococcus avium, Eubacterium_sp_3_1_31*, and

Clostridium hatheway; (3) *Streptococcus gordonii*, *Enterococcus avium*, and *Eubacterium_sp_3_1_31*; or (4) *Streptococcus gordonii* and *Enterococcus avium*. In some examples, the reagents comprise a set of oligonucleotide primers for amplification of a polynucleotide sequence from any one of the bacterial species set forth in Table 8 or 9. In some examples, the amplification is PCR, for example, quantitative PCR (qPCR).

[0018] In a sixth example, the present disclosure provides a method for promoting growth and development of a child, comprising administering to the child an effective amount of one or more bacterial species selected from Table 8. In some examples, the child is between about 3 to about 6 years old in biological age.

[0019] In a related example, a kit is provided for promoting growth and development of a child. The kit includes a first container containing a first composition comprising (i) an effective amount of one of the bacterial species set forth in Table 8 and a second container containing a second composition comprising (i) an effective amount of another, different one of the bacterial species set forth in Table 8. In some examples, the first or second composition comprises processed donor fecal material for FMT. In some examples, the first or second composition is formulated for oral administration. In some examples, both the first and second compositions are formulated for oral ingestion.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] **Figure 1.** Differential bacterial species between autism spectrum disorder children and typically developing children. The green bars represent species enriched in typically developing children, whereas the red bars represent species enriched in ASD children.

[0021] **Figure 2**. Receiver operating characteristic (ROC) curve and the area under the curve (AUC) of the machine learning model. AUC of random forest model using: Top 1 marker (red, bottom line) - *Alistipes indistinctus*. Top 3 markers (green, middle line) - *Alistipes indistinctus, candidate division TM7 single-cell isolate TM7c*, and *Streptococcus cristatus*. All 5 markers (dark blue, top line) - *Alistipes indistinctus, candidate division TM7 single-cell isolate TM7c, Streptococcus cristatus, Eubacterium_limosum*, and *Streptococcus_oligofermentans*.

[0022] **Figure 3.** Risk score of a 3-year-old child compared to ASD children and typically developing children using (a) 5 markers: *Alistipes indistinctus, candidate division TM7 single-cell isolate TM7c, Streptococcus cristatus, Eubacterium_limosum*, and

WHAT IS CLAIMED IS:

1. A method for assessing risk for autism spectrum disorder (ASD) in a human child, comprising:

(1) determining, in a stool sample which is to be obtained from the child, the level or relative abundance of one or more of the bacterial species set forth in Table 3;

(2) determining the level or relative abundance of the same bacterial species in a stool sample from a reference cohort comprising normal and ASD children;

(3) generating decision trees by random forest model using data obtained from step (2) and running the level or relative abundance of one or more of the bacterial species from step (1) down the decision trees to generate a risk score; and

(4) determining the child with a risk score greater than 0.5 as having an increased risk for ASD and determining the child with a risk score no greater than 0.5 as having no increased risk for ASD.

2. The method of claim **28**, wherein the one or more bacterial species comprise *Alistipes indistinctus*.

3. The method of claim **28**, wherein the one or more bacterial species comprise *Alistipes indistinctus, candidate division TM7 single-cell isolate TM7c*, and *Streptococcus cristatus*.

4. The method of claim **28**, wherein the one or more bacterial species comprise *Alistipes indistinctus, candidate division TM7 single-cell isolate TM7c, Streptococcus cristatus, Eubacterium_limosum*, and *Streptococcus_oligofermentans*.

5. A method for treating symptoms of autism spectrum disorder (ASD) in a human child, comprising introducing into the child's gastrointestinal tract an effective amount of one or more of the bacterial species of *Faecalibacterium prausnitzi*, *Roseburia inulinivorans*, *Eubacterium hallii*, *Dorea longicatena*, or *Eubacterium siraeum*.

6. A method for treating symptoms of autism spectrum disorder (ASD) in a human child, comprising reducing the level or relative abundance of one or more of the bacterial species of *Clostridium nexile*, *Dialister invisus*, *Clostridium bolteae*, *Clostridium symbiosum*, *Eubacterium limosum*, *Clostridiales bacterium_1_7_47FAA*, *Clostridium*

ramosum, *Anaerotruncus colihominis*, *Clostridium citroniae*, or *Alistipes indistinctus* in the child's gastrointestinal tract.

7. A kit for treating symptoms of ASD, comprising: a first container containing a first composition comprising (i) an effective amount of one of the bacterial species set forth in Table 1, or (ii) an effective amount of an anti-bacterial agent that suppresses growth of one of the bacterial species set forth in Table 2, and a second container containing a second composition comprising (i) an effective amount of another one of the bacterial species set forth in Table 1, or (ii) an effective amount of an anti-bacterial agent that suppresses growth of another one of the bacterial species set forth in Table 2.

8. A method for determining risk for autism spectrum disorder (ASD) in a human child, comprising:

(1) determining, in a stool sample from the child, the relative abundance of any one of the bacterial species set forth in Table 1 or 2; and

(2) detecting the relative abundance from step (1) being no lower than the cutoff value in Table 1 or a standard control value or being lower than the cutoff value or a standard control value in Table 2 and determining the child as not having increased risk for ASD; or detecting the relative abundance from step (1) being lower than the cutoff value in Table 1 or a standard control value or being no lower than the cutoff value in Table 2 or a standard control value and determining the child as having an increased risk for ASD.

9. A method for assessing risk for autism spectrum disorder (ASD) in two human children, comprising:

(1) determining, in a stool sample from each of the two children, the relative abundance of any one of the bacterial species set forth in Table 1 or 2;

(2) determining the relative abundance of a bacterial species set forth in Table 1 from step (1) being higher in the stool sample from the first child or the relative abundance of a bacterial species set forth in Table 2 from step (1) being lower in the stool sample from the first child; and

(3) determining the second child as having a higher risk for ASD than the first child.

10. A method for determining risk for autism spectrum disorder (ASD) in a human child, comprising:

(1) obtaining in a stool sample from the child a value of (a) the relative abundance of *Alistipes indistinctus* (Ai) or *Anaerotruncus colihominis* (Ac), or (b) the combined score of levels of three bacterial species Ai, Ac, and *Eubacterium hallii* (Eh), which is calculated by by $I1 + \beta1*Ai + \beta2*Eh + \beta3*Ac$; and

(2) detecting the value to be higher than a standard control value and determining the individual as having increased risk of ASD.

11. A method for determining risk for autism spectrum disorder (ASD) in a human child, comprising:

(1) obtaining in a stool sample from the child a value of the relative abundance of *Eubacterium hallii* (Eh); and

(2) detecting the value to be lower than a standard control value and determining the individual as having increased risk of ASD.

12. A kit for assessing risk for autism spectrum disorder (ASD), comprising reagents for detecting one or more of the bacterial species set forth in Table 1, 2, or 3.

13. A method for determining developmental age of a child, comprising the steps of:

(a) quantitatively determining the relative abundance of one or more bacterial species selected from Table 8 or 9 in a stool sample taken from the child;

(b) quantitatively determining the relative abundance of the one or more bacterial species in a stool sample taken from a reference cohort consisting of typically developing children;

(c) generating decision trees by random forest model using data obtained from step (b); and

(d) running the relative abundances obtained from step (a) down the decision trees from step (b) to generate a developmental age for the child.

14. A kit for determining developmental age of a child, comprising: a first container containing a first reagent for detecting a first bacterial species set forth in Table 8 or 9, and a second container containing a second reagent for detecting a second bacterial species set forth in Table 8 or 9.

15. The kit of claim **42**, comprising two or more containers each of which containing a reagent for detecting a different bacterial species selected from the group consisting of (1) *Streptococcus gordonii*, *Enterococcus avium*, *Eubacterium_sp_3_1_31*, *Clostridium hathewayi*, and *Corynebacterium durum*; (2) *Streptococcus gordonii*, *Enterococcus avium*, *Eubacterium_sp_3_1_31*, and *Clostridium hatheway*; (3) *Streptococcus gordonii*, *Enterococcus avium*, and *Eubacterium_sp_3_1_31*; or (4) *Streptococcus gordonii* and *Enterococcus avium*.

16. A method for promoting growth and development of a child, comprising administering to the child an effective amount of one or more bacterial species selected from Table 8.

17. A kit for promoting growth and development of a child, comprising: a first container containing a first composition comprising (i) an effective amount of one of the bacterial species set forth in Table 8, and a second container containing a second composition comprising (i) an effective amount of another one of the bacterial species set forth in Table 8.