A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial

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BACKGROUND: Recent experimental evidence suggests that gut microbiota may alter function within the nervous system providing new insight on the mechanism of neuropsychiatric disorders.

METHODS: Seventy-five infants who were randomized to receive *Lactobacillus rhamnosus* GG (ATCC 53103) or placebo during the first 6 mo of life were followed-up for 13 y. Gut microbiota was assessed at the age of 3 wk, 3, 6, 12, 18, 24 mo, and 13 y using fluorescein in situ hybridization (FISH) and qPCR, and indirectly by determining the blood group secretor type at the age of 13 y. The diagnoses of attention deficit hyperactivity disorder (ADHD) and Asperger syndrome (AS) by a child neurologist or psychiatrist were based on ICD-10 diagnostic criteria.

RESULTS: At the age of 13 y, ADHD or AS was diagnosed in 6/35 (17.1%) children in the placebo and none in the probiotic group ($P = 0.008$). The mean (SD) numbers of *Bifidobacterium* species bacteria in feces during the first 6 mo of life was lower in affected children 8.26 (1.24) log cells/g than in healthy children 9.12 (0.64) log cells/g; $P = 0.03$.

CONCLUSION: Probiotic supplementation early in life may reduce the risk of neuropsychiatric disorder development later in childhood possible by mechanisms not limited to gut microbiota composition.

Psychiatric disorders are already ranked among the leading causes of disability in industrialized countries. With the current progressive increase in the incidence, they may be expected to assume the first place also globally within the next few years (1,2). Attention-deficit hyperactivity disorder (ADHD), characterized by inattention, impulsivity, and hyperactivity, affects three to seven percent of children worldwide (3,4). Moreover, symptoms of inattention and hyperactivity are frequent in children with Asperger syndrome (AS), which is characterized by stereotyped behavior and deficient social interaction and communication skills (5). Besides the common behavioral features, shared biological pathways and neuroanatomical links between these diseases have been reported (5,6).

Despite intensive research on ADHD and AS, the precise chain of pathological events underlying them remains unknown. The available data indicate ADHD and AS to be multifactorial disorders, in which genetic risk predominates, reinforced by various environmental and biological factors such as fetal stress, prematurity, toxins, and diet (7). Recently, the search for etiologies has been expanded both within the central nervous system and beyond. Experimental data are accumulating to suggest that the presence of gut microbiota as such, as compared with the absence of it, and especially its certain beneficial bacteria, probiotics, make for altered function within the nervous system (8–12). As a recent empirical study indicates (13) probiotics may provide a tool to manipulate brain activity even in humans.

To test the hypothetical involvement of the gut brain-axis in the manifestation of ADHD and AS, we analyzed the association of compositional development of the gut microbiota, the blood group secretor type as an indirect evidence of gut microbiota involvement, and the impact of specific probiotic intervention on the emergence of these two neuropsychiatric disorders in a cohort followed until 13 y of age.

RESULTS

The 13-y follow-up was completed by 75 of the study subjects; 40 (53.3%) of these initially received *Lactobacillus rhamnosus* GG and 35 (46.7%) placebo (**Figure 1**). Clinical characteristics of the study population were comparable between the intervention groups (**Table 1**). Furthermore, all the clinical characteristics, except for length of exclusive breast-feeding, were comparable between the children who did and did not complete the 13-y follow-up (**Table 2**).

ADHD was diagnosed in three (4.0%) children, AS in one (1.3%) child, and both ADHD and AS in two (2.7%) by the age of 13 y. Of these six children, four were on methylphenidate and two on daily ω-3 fatty acid supplementation. All the children with neuropsychiatric diagnoses were male. ADHD or AS had been diagnosed in 6/35 (17.1%) children in the placebo and none in the probiotic group by the age of 13 y ($P = 0.008$). When logistic regression analysis was controlled

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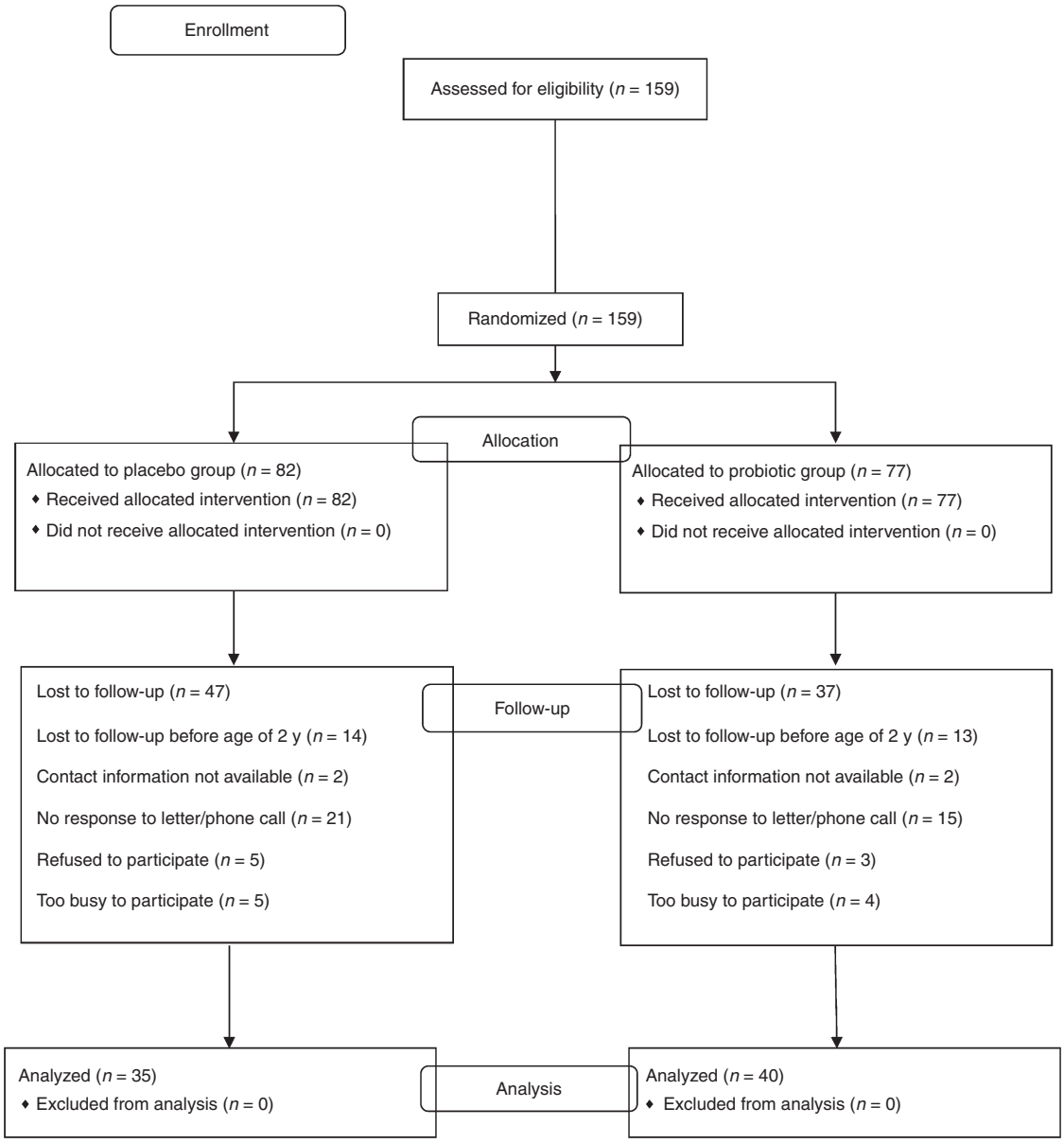

Figure 1. Flow chart of the procedure.

for gender, the result remained significant ($P = 0.02$). All the other clinical characteristics, as shown in **Table 3**, were comparable between healthy children and those with ADHD or AS. Two children affected by AS were nonsecretors (33% of AS/ADHD children), while in nonaffected children this was the case in 12% ($P = 0.20$).

During the first 3 mo of life, the differences in gut microbiota composition between children who later developed ADHD or AS compared to healthy children were not manifested in bacterial numbers as analyzed by FISH (**Figure 2**), but in species composition of *Bifidobacterium*, assessed by qPCR. The distinction was due to significantly lower median (IQR) numbers of *Bifidobacterium longum* at the age of 3 mo among the children with neuropsychiatric disorders than in healthy children (4.35 (3.99–10.40) log/g and 10.18 (8.88–10.88) log/g, respectively, $P = 0.045$).

At the age of 6 mo, at weaning, when probiotic intervention was completed, the mean (SD) number of cells belonging to the genus *Bifidobacterium* as measured with FISH was significantly lower among children with neuropsychiatric disorders than in those without (8.26 (1.24) log cells/g vs. 9.12 (0.64) log cells/g, respectively, $P = 0.03$). At the age of 18 mo, the mean (SD) numbers of *Bacteroides* and *Lactobacillus-Enterococcus* group bacteria were lower among children with ADHD/AS than healthy children (7.28 (0.85) log cells/g vs. 8.13 (0.51) log cells/g, $P = 0.008$; 7.71 (0.78) log cells/g vs. 8.40 (0.40) log cells/g, $P = 0.01$, respectively (**Figure 2**). Furthermore, at the age of 24 mo, the mean (SD) numbers of cells belonging to the *Clostridium histolyticum* group were lower among children with ADHD or AS than in healthy children, (7.46 (0.44) log cells/g vs. 8.16 (0.55) log cells/g; $P = 0.04$).

Table 1. Clinical characteristics of the study subjects

	Probiotic, $n = 40$	Placebo, $n = 35$
Male	24 (60)	16 (46)
Vaginal delivery	33 (83)	29 (83)
Gestational age at birth (wk)[a]	40 (2)	40 (2)
Birth weight (g)[a]	3,493 (573)	3,694 (574)
Birth length (cm)[a]	51 (2)	51 (2)
Apgar score at 5 min[a]	9 (1)	9 (1)
Exclusively breast-fed (months)[a]	3(2)	3 (2)
Antibiotic treatment during the first 6 mo of life	13 (33)	7 (20)
Weight at age of 13 y (kg)[a]	56 (12)	57 (12)
Length at age of 13 y (cm)[a]	165 (9)	165 (9)
Systolic blood pressure at the age of 13 (mmHg)[a]	104 (10)	106 (11)
Waist circumference (cm)[a]	70 (8)	72 (10)

Results are given as mean (SD)[a] or as number (%) of subjects. Clinical characteristics of the study population were comparable between the groups ($P > 0.05$ in all the comparisons).

Table 2. Clinical characteristics of the children completing and not completing the present study

	Children completing the study, $n = 75$	Dropouts, $n = 83$
Male	40 (53)	53 (64)
Vaginal delivery	62 (83)	70 (84)
Gestational age at birth (wk)[a]	40 (2)	40 (1)
Birth weight (g)[a]	3,585 (582)	3,631 (435)
Birth length (cm)[a]	51 (2)	51 (2)
Apgar score at 5 min[a]	9 (1)	9 (1)
Exclusively breast-fed (months)[a]	3(2)	2 (2)
Antibiotic treatment during the first 6 mo of life	20 (27)	16 (24)

Results are given as mean (SD)[a] or as number (%) of subjects. Clinical characteristics of the study population were comparable between the groups ($P > 0.05$ in all the comparisons), except for length of exclusive breast-feeding ($P = 0.03$).

At the age of 13, there were no statistically significant differences in gut microbiota composition, analyzed with FISH or qPCR, between children with or without neuropsychiatric disorders (**Figure 2**).

DISCUSSION

The results of our preliminary study demonstrate for the first time that specific probiotics may reduce the risk of the development of ADHD and AS possibly by mechanisms not directly associated with gut microbiota composition, since no single constant microbiota composition or their difference could be distinctive in children with or without neuropsychiatric disorders.

These findings must be viewed in the light of some caution as preliminary and initial observation into this novel area. First, our probiotic intervention study was originally designed and statistically powered for prevention of atopic eczema, not for prevention of more uncommon neuropsychiatric disorders ADHD and AS. Regardless of that, it is interesting to notice that allergic disease has been shown to be associated with an increased risk of ADHD and differences in neurodevelopment (14,15) suggesting a possibility for common environmental determinants. Second, the number of drop-outs was quite considerable during the follow-up. Therefore, we cannot discount the possibility that the issue would not have biased our findings. However the number of drop-outs was equally divided in the both intervention groups and base-line characteristics of the drop-outs and the study finishers were similar, except for the duration of exclusive breast-feeding, demonstrating that these two groups were unbiased in almost all of the known factors. On the other hand, the careful and prospective 13-y follow-up period is the strength of the study. A further strength of the study is that gut microbiota has been analyzed comprehensively by FISH and qPCR as well as indirectly by blood group secretor type analysis.

Table 3. Clinical characteristics of children with and without ADHD or AS

	Healthy, $n = 69$	ADHD/AS, $n = 6$	P value
Early probiotic supplementation	40 (58)	0 (0)	$P = 0.008$
Male	34 (49)	6 (100)	$P = 0.03$
Vaginal birth	58 (84)	4 (67)	$P = 0.28$
Exclusively breast-fed (months)[a]	3 (2)	2 (2)	$P = 0.16$
Antibiotic treatment during the first 6 mo of life	19 (28)	1 (17)	$P = 1.00$
Total distress during the 7th week (min/day)[a,b]	107 (73)	101 (87)	$P = 0.87$
Total distress during the 12th week (min/day)[a,c]	68 (46)	50 (50)	$P = 0.42$
Weight at age of 13 y (kg)[a]	54 (12)	57 (12)	$P = 0.64$
Length at age of 13 y (cm)[a]	165 (9)	163 (10)	$P = 0.48$
Systolic blood pressure at age of 13 y (mmHg)[a]	105 (10)	105 (14)	$P = 0.92$

Results are given as mean (SD)[a], or as numbers (%) of subjects. [b]The sum of colic type, other cry, and fussing reported by parents; $n = 47$ for healthy and $n = 5$ for ADHD/AS. [c]The sum of colic type, other cry, and fussing reported by parents; $n = 45$ for healthy and $n = 5$ for ADHD/AS.

ADHD is a disease of substantial genetic predisposition, as suggested by a number of adoption, twins, and family studies (7,16,17). This notwithstanding, recent studies document up to 20% discordance in identical twins, leaving room for environmental and epigenetic determinants of ADHD. Thus far, prenatal exposure to maternal smoking (18), low birth weight, prematurity (19,20), and specific environmental exposures such as lead and organic pollutants (7) have been linked to ADHD. In contrast, research on dietary factors such as sugar, artificial food colorings, zinc, iron, and ω-3 fatty acids, has established only vague causality in ADHD (7), despite some hints of their therapeutic potential (21,22). An empirical elimination diet has indeed been shown to be effective in

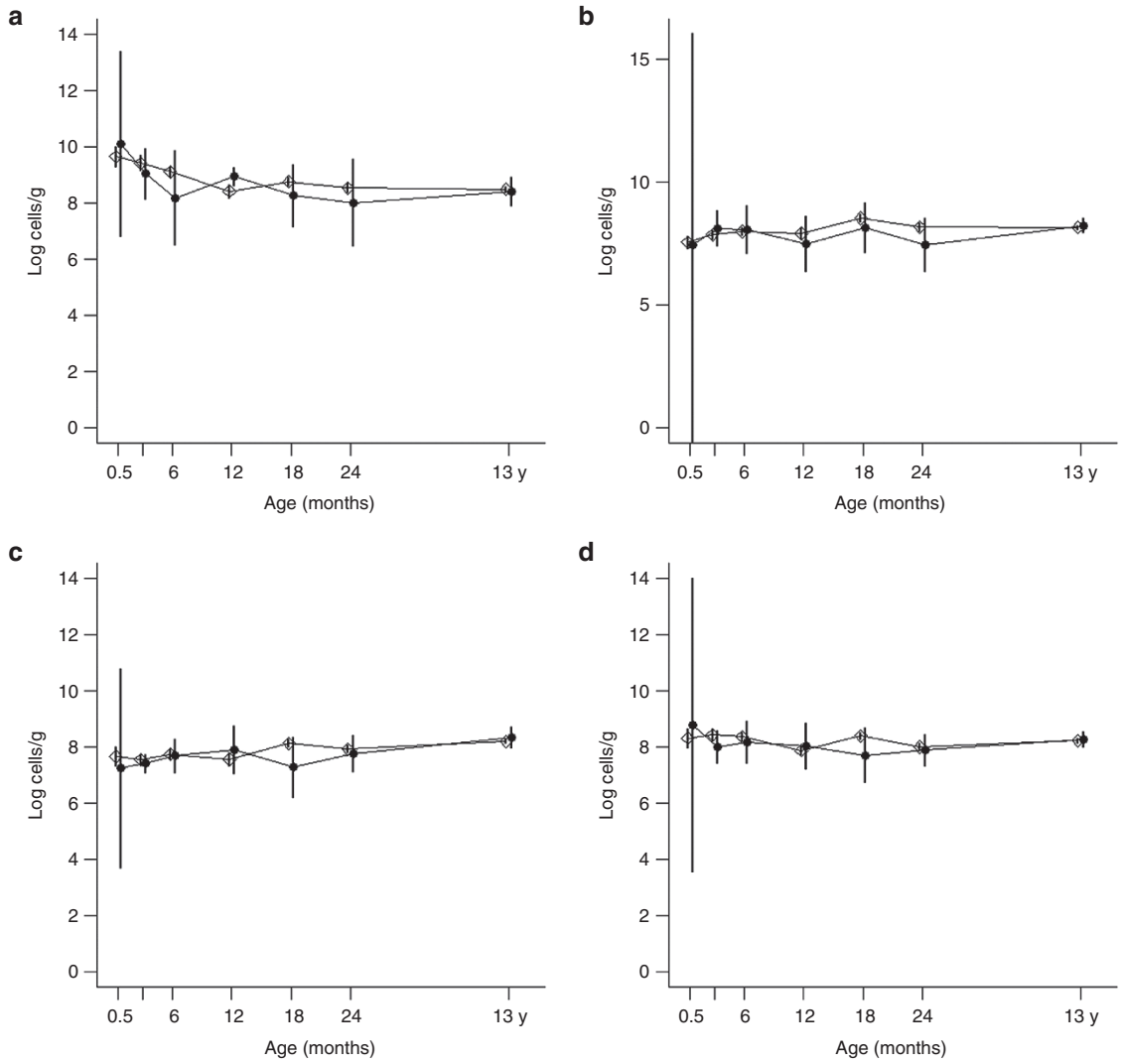

Figure 2. The gut microbiota composition (mean (95% CI) log cells/g) development analyzed by FISH during the first 13 y of life among children with (circle) and without (diamond) neuropsychiatric disorders. **a**, *Bifidobacterium*; **b**, *Clostridium histolyticum*; **c**, *Bacteroides-Prevotella*; **d**, *Lactobacillus-Enterococcus*. Number of fecal samples analyzed in children with neuropsychiatric disorders: $n = 2$ at 0.5 mo, $n = 4$ at 3 mo, $n = 5$ at 6, 12, and 18 mo, $n = 3$ at 24 mo, $n = 4$ at 13 y. Number of samples analyzed in healthy children: $n = 19$ at 0.5 mo, $n = 41$ at 3 mo, $n = 53$ at 6 mo, $n = 59$ at 12 mo, $n = 55$ at 18 mo, $n = 48$ at 24 mo, $n = 53$ at 13 y.

the treatment of ADHD (22). Moreover, breastfeeding, as a potent inducer of *Bifidobacteria* in the gut microbiota (23), has proved to be associated with lower levels of conduct disorder symptoms in middle childhood, which is in line with our findings in this study (24) demonstrating defiencies in early *Bifidobacterium* composition in those with later ADHD or AS.

Although the precise possible mechanisms of action of the gut microbiota in the gut-brain axis are unclear, a previous experimental study offers an interesting clue. In the study in question, mice with experimentally induced chronic colitis showed anxiety-like behavior. Treatment with *Bifidobacterium longum* abolished such behavior. However, the anxiolytic effect of *Bifidobacterium longum* was absent in vagotomized mice, suggesting that the effect was transmitted to the central nervous system by activating vagal pathways at the level of the enteric nervous system (9). It is thus intriguing to note that in our study the amount of the same species was found to be decreased in early life in children later developing ADHD or AS.

Our data demonstrate that early administration of *Lactobacillus rhamnosus* GG may reduce the risk of ADHD and AS. *Lactobacillus rhamnosus* GG has been shown to stabilize the gut permeability barrier by effects on tight junctions, mucin production and antigen-specific immunoglobulin A production (25). In addition, a recent experimental study has demonstrated that *Lactobacillus rhamnosus* regulates, again via the vagus nerve, emotional behavior and the central GABAergic system, which is also associated with neuropsychiatric disorders (11,26). Of note, a recent study with healthy women demonstrated that a consumption of a mixture of probiotic bacteria had significant effects on brain regions that control central processing of emotion and sensation (13). Probiotics had no significant effect on microbiota composition in the study suggesting that the effects on central nervous

system were either induced by altered vagal afferent signaling or by systemic metabolic changes related to probiotic intake (13). Furthermore, another experimental study with mice showed that pretreatment with probiotic *Lactobacillus rhamnosus* prevents learning and memory dysfunction in *Citrobacter rodentium*-infected mice (27). It thus remains to be elucidated whether these effects of *Lactobacillus rhamnosus* are of importance in the development of neuropsychiatric disorders in humans. However, in accord with our clinical findings here it has been postulated that neural pathways may alter already early in development. If such an alteration takes place at a critical moment, the sequential dysfunction of the gut-brain axis may become relatively constant into adulthood (28,29).

Our findings demonstrate a possible preventive risk reducing effect of a probiotic LGG on later development of ADHD and AS. We also report an interconnection between the early gut microbiota and development of these neuropsychiatric disorders, although no single constant microbiota composition component or change was detected. However, keeping in mind the above-mentioned limitations of the study, we consider the findings preliminary but encouraging for further studies of the subject both in the area of well-powered clinical trials and experimental research.

METHODS

Ethics Statement

The study was approved by the committees on ethical practice in Turku University Hospital and the Health Office of Turku. Written informed consent was obtained from the children and their parents.

Study Population and Design

The subjects included in this study are participants in an ongoing, randomized, double-blind, placebo-controlled prospective follow-up study involving perinatal *Lactobacillus rhamnosus* GG (ATCC 53103) intervention (http://www.clinicaltrials.gov/ct/gui/show/NCT00167700) as described in detail elsewhere (30,31). In brief, the original study population comprised 159 infants who had at least one family member with allergic disease. The mothers of these children were recruited in the antenatal clinics of the city of Turku between February 1997 and January 1998 and randomized in double-blind, placebo-controlled manner to receive $1x10^{10}$colony-forming units of *Lactobacillus rhamnosus* GG or placebo (microcrystalline cellulose) daily for 4 wk before expected delivery. After delivery, the capsule contents were given either to the children, or continuously to the mothers, if breast-feeding, for 6 mo.

Clinical Evaluation

All the 132 children (83% of the original cohort) completing the 2-y follow-up were invited to the 13-y follow-up visit. During the study visit between June 2011 and October 2011, a clinical examination was carried out by AP. Weight, height, mid-upper arm and waist circumference, and blood pressure were measured. Data were prospectively collected by the parents throughout the follow-up on breast-feeding, acute and chronic conditions, antibiotics and other medications, and probiotics and other diet. Data were also collected from the records of well-baby clinics and hospital.

Assessment of Early Behavior Patterns

During the 7th and 12th week of life parents recorded their infants' behavior patterns (sleeping time, time awake and content, fussing, colic-type cry, other cry, and feeding), vomits, stools with their consistency, and skin condition, using a modified 24-h Barr chart (32) on 7 consecutive days (31,32). Fussing was defined as a state of irritability, "not quite crying but not awake and content". Other cry was defined as crying responsive to intervention (feeding, diaper change, carrying, sucking a pacifier), and colic-type cry as crying not responsive to such intervention. Total distress, the amount of crying and fussing, was the sum of these various modes as reported by the parents.

Assessment of Neuropsychiatric Disorders

The clinical diagnosis of ADHD and AS was made by an experienced child psychiatrist or neurologist not involved in the study or follow-up and blinded with randomization. The diagnostic criteria of the International Classification of Diseases (ICD-10) were used. Accordingly, a person with ADHD must have at least six of the listed symptoms of inattention and at least three of the listed symptoms of hyperactivity and impulsivity, these having persisted for at least 6 mo to a degree that is maladaptive and inconsistent with the person's development level. The symptoms must have begun before the age of 7 y, must present in at least two places, negatively affect school, social or occupational functioning, and are not better explained by an alternative disorder. According to the ICD-10, a person with Asperger syndrome shows no clinically significant general delay in spoken or receptive language or cognitive development but has qualitative abnormalities in reciprocal social interaction and unusually intense circumscribed interest or restrictive, repetitive and stereotyped patterns of behavior, interests, and activities. The disorder is not attributable to other varieties of pervasive developmental disorder.

Fecal Samples

A fecal sample was taken at 3 wk, 3, 6, 12, 18, 24 mo, and 13 y either by nursing staff at a scheduled visit or by the parents/children immediately before the visit. In the latter case, the sample was stored at 4 °C and delivered to the hospital within 24 h.

DNA Isolation and qPCR Assay

Fecal samples were stored at −80 °C until analyzed. They were pretreated and DNA was extracted using an automated KingFisher DNA extraction system (Thermo Fisher Scientific Oy, Vantaa, Finland) and InviMag Stool DNA kit (Stratec Molecular, Berlin, Germany), as previously described (33). The standard DNA for quantitative PCR (qPCR) was prepared as described elsewhere (33). All DNA samples were stored at −20 °C until analyzed. Quantitative PCRs were conducted as previously described (34,35). PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Foster City, CA).

FISH Assay

Bacterial cells were harvested and fixed and FISH carried out with fluorophore (indocarbocyanine Cy3)-labeled oligonucleotide probes, as previously described (36). Total cell numbers were determined by nucleic acid stain 4′, 6-diamidino-2-phenylindole (DAPI). Cells were counted visually using an Olympus SZX9 epifluorescence microscope (Olympus Optical Co LTD, Tokyo, Japan).

Blood Group Secretor Status

At the age of 13 y, histo-ABO blood group secretor status was determined by genotyping a single-nucleotide polymorphism (SNP) rs601338 (W143X, G428A) of FUT2 gene. Nonsecretor individuals have a nonsense mutation in rs601338 (genotype AA) leading to unfunctional fucosyltransferase 2 enzyme, and therefore the nonsecretors do not express histo-ABO blood group antigens in intestinal mucosa or other secretions. The SNP rs601338 determines the nonsecretor status in Finnish population (37). The SNP rs601338 was genotyped by using Taqman chemistry (Life Technologies, Carlsbad, CA) (fwd primer: GGGAGTACGTCCGCTTCAC, rev primer: TGGCGGAGGTGGTGGTA, reporter 1-VIC: CTGCTCCTAGACCTT, reporter 2-FAM: CTGCTCCTGGACCTT) on LightCycler 480 Real-Time PCR Instrument (Roche, Switzerland) in the Institute for Molecular Medicine Finland (FIMM) as previously described (38).

Statistical Methods

The clinical characteristics of the study subjects for continuous variable are given as mean values with SD, and as a number and proportions for categorical variables. Comparisons between two continuous variables were made using *t*-test or Mann–Whitney *U*-test, as appropriate. Comparisons between two categorical variables were made using χ^2 test or Fisher's exact test, as appropriate. Univariate associations between the response variable (neuropsychiatric disorders)

and continuous predictor variables were studied using logistic regression analysis. In all PCR assays, except with *Bifidobacterium genus* and *Bifidobacterium longum*, a large proportion of observations were below the detection limit. These observations were dichotomized as either above or below the detection limit for statistical analyses. In all other microbial assays, the proportion of observations below detection limit was at most seven percent. These observations were excluded from statistical analyses. Statistical analyses were made using SAS for Windows version 9.2. *P* values below 0.05 were considered statistically significant.

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