



Rapid Assessment of Microbiota Changes in Individuals with Autism Spectrum Disorder Using Bacteria-derived Membrane Vesicles in Urine

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Individuals with autism spectrum disorder (ASD) have altered gut microbiota, which appears to regulate ASD symptoms via gut microbiota-brain interactions. Rapid assessment of gut microbiota profiles in ASD individuals in varying physiological contexts is important to understanding the role of the microbiota in regulating ASD symptoms. Microbiomes secrete extracellular membrane vesicles (EVs) to communicate with host cells and secreted EVs are widely distributed throughout the body including the blood and urine. In the present study, we investigated whether bacteria-derived EVs in urine are useful for the metagenome analysis of microbiota in ASD individuals. To address this, bacterial DNA was isolated from bacteria-derived EVs in the urine of ASD individuals. Subsequent metagenome analysis indicated markedly altered microbiota profiles at the levels of the phylum, class, order, family, and genus in ASD individuals relative to control subjects. Microbiota identified from urine EVs included gut microbiota reported in previous studies and their up- and down-regulation in ASD individuals were partially consistent with microbiota profiles previously assessed from ASD fecal samples. However, overall microbiota profiles identified in the present study represented a distinctive microbiota landscape for ASD. Particularly, the occupancy of *g_Pseudomonas*, *g_Sphingomonas*, *g_Agrobacterium*, *g_Achromobacter*, and *g_Roseateles* decreased in ASD, whereas *g_Streptococcus*, *g_Akkermansia*, *g_Rhodococcus*, and *g_Halomonas* increased. These results demonstrate distinctively altered gut microbiota profiles in ASD, and validate the utilization of urine EVs for the rapid assessment of microbiota in ASD.

Key words: Autism spectrum disorder, gut microbiota, Extracellular membrane vesicles, Bacteria-derived EVs, urine marker

INTRODUCTION

Autism spectrum disorder (ASD) is a group of neurodevelopmental disabilities characterized by two domains of core symptoms, persistent social deficits and restricted repetitive patterns of behavior [1]. Most individuals with ASD suffer from various behavioral and physical symptoms, including abnormal preferences regarding specific foods and problems in the digestive system [2, 3].

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Approximately 70–80% of ASD subjects have food selectivity and restricted food interests due to the texture, smell, or color of specific foods, and food intolerance [3, 4]. The limited food intake behavior in ASD subjects leads to health problems including nutrition imbalance and gastrointestinal (GI) symptoms, such as diarrhea and constipation [2, 5, 6]. Furthermore, studies of a positive correlation between GI symptoms and ASD have been reported; the c-Met promoter variant rs1858830 is associated with ASD and GI symptoms, and the serum level of hepatocyte growth factor (HGF) that binding to the c-Met receptor, is correlated with severity of GI symptom in ASD subjects [7–9].

Several lines of evidence indicate that ASD patients have altered microbiota composition in the gut compared to healthy subjects [10–21]. The occupancy of the phyla *Firmicutes*, *Fusobacteria*, *Verrucomicrobia*, and *Actinobacteria* was decreased, whereas *Bacteroidetes* and *Proteobacteria* were increased in ASD groups [13, 21]. More specifically, in ASD, the genera *Bifidobacterium* and *Akkermansia* were found to be decreased in ASD, while *Lactobacillus* was increased [17, 18]. Furthermore, the treatment with a probiotics mix containing *Streptococcus (thermophiles)*, *Bifidobacterium breve*, *B. longum*, *B. infantis*, and *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii (subsp. Bulgaricus)* or the transplant of fecal microbiota from healthy subjects to ASD individuals increased overall bacterial diversity and the abundance of *Bifidobacterium*, *Prevotella*, and *Desulfovibrio* among other taxa, and alleviated GI symptoms and ASD core symptoms [22, 23]. To date, all available microbiota composition in ASD were mostly assessed from fecal samples [11, 13, 14, 17–21] or directly from the cecum and ileum [15, 16]. The fact that some microbiota commonly change in independent studies, and others are not consistently reported (e.g., [13, 15, 19]), increases the possibility of highly complex dynamics in bodily microbiota composition in ASD individuals under different physiological contexts.

Gram-negative bacteria secrete extracellular membrane vesicles (EVs), also called nanovesicles, to communicate with host cells [24], and are detected in stools, and also in urine and blood serum [25–27]. EVs secreted by gram-negative bacteria contain DNA, RNA, proteases, phospholipases, adhesins, toxins, and immunomodulatory compounds. Bacteria-derived EVs are associated with cytotoxicity, bacterial attachment, intercellular DNA transfer, and invasion [24, 28, 29]. Gram-positive bacteria also produce EVs, which contain peptidoglycan, lipoteichoic acid, virulence proteins, DNA and RNA [30, 31]. When bacterial EVs were intraperitoneally injected in mice, EVs were rapidly distributed throughout the body with accumulation in the liver, lung, spleen, and kidney within 3 h [27]. Bacteria-derived EVs in the blood and urine represent the major constituents of microbes in the body, namely the gut micro-

biota [25, 26], and indicate the microbiota that are metabolically or pathologically active [25, 27].

In the present study, we investigated bodily microbiota represented by bacterial EVs in the urine of ASD individuals. The results of the present study identify markedly altered microbiota profiles in ASD relative to non-ASD healthy controls and suggest that bacterial EVs in urine can be served as a useful tool for the evaluation of microbiota composition in ASD.

MATERIALS AND METHODS

Subjects and urine sample preparation

Individuals who were enrolled at the Ewha Special Education Research Institute (Seoul, Republic of Korea) or Ewha Womans University MokDong Hospital (Seoul, Republic of Korea) were diagnosed according to the DSM-5 diagnostic criteria by a child and adolescent psychiatrist followed by characterization using the Korean Childhood Autism Rating Scale (K-CARS) as described previously [32]. The K-CARS is a well-established scale for the diagnosis of ASD with good agreement with the DSM-5 diagnostic criteria [33]. This questionnaire contained 15 items, each with 4 symptom scales, and all individual scores on each of the questions were summed to obtain the total score. When the total score was higher than 30 points, the subject was classified as autistic. Individuals who had any associated additional psychiatric and neurological diagnoses, or individuals who were on any antipsychotic medications were excluded from the present study.

Among the characterized ASD individuals, 18 male and 2 female ASD individuals (22.4±/–4.9 years) (Table 1) were joined to this study and their urine was collected during the day. The collected urine samples were frozen and stored at –20°C until use. Age-matched normal healthy subjects (24 males and 4 females, 21.1±/–9.5 years) (Table 1) were selected from the Inje University Haeundae Paik Hospital (IRB No. 1297992-2015-064) and Seoul National University Hospital Healthcare System Gangnam Center (IRB No. 1502-034-647). The control subjects were not related to ASD and had no clinical findings suggestive of gastrointestinal problems or neuropsychiatric disorders. The control subjects of this study had not taken antibiotics, probiotics or prebiotics in the 3 months prior to the sample collection.

Table 1. The number, age, and sex of control and ASD subjects

	Control	ASD	p-value
Age (years)	21.1±9.5	22.4±4.9	0.57
N	28	20	-
Sex (Male/Female)	24/4	18/2	0.66

The experimental protocol of human subjects was reviewed and approved by the Institutional Review Board of Ewha Womans University Hospital (IRB No. 2015-08-005-002). All eligible participants had been told about the purpose, procedures, risks and benefits of the present study and informed consent was obtained from all ASD subjects.

Isolation of bacteria-derived EVs and DNA extraction from human urine samples

Bacteria EVs were isolated from the urine of ASD individuals following the procedure described previously [25, 26]. Briefly, each urine sample was centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant was taken and passed through a $0.22\text{-}\mu\text{m}$ membrane filter to eliminate foreign particles. Isolated EVs were dissolved in $100\ \mu\text{l}$ PBS, and quantified on the basis of protein.

Bacterial DNA extraction from prepared EVs was performed as described previously [25, 26]. Briefly, isolated EVs ($1\ \mu\text{g}$ by protein, each sample) were boiled at 100°C for 40 min, centrifuged at $13,000\ g$ for 30 min, and the supernatants were collected. Collected samples were then subjected to bacterial DNA extraction using a DNA extraction kit (PowerSoil DNA Isolation Kit, MO BIO, USA) following the manufacturer's instructions. Isolated DNA was quantified by using the QIAxpert system (QIAGEN, Germany).

PCR amplification, library construction, and sequencing of 16S rRNA gene variable regions

Prepared bacterial DNA was used for PCR amplification of the V3-V4 hypervariable regions of the 16S ribosomal RNA genes using the primer set of 16S_V3_F ($5'\text{-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'}$) and 16S_V4_R ($5'\text{-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'}$). The PCR products were used for the construction of 16S rDNA gene libraries following the MiSeq System guidelines (Illumina Inc., San Diego, CA, USA). The 16S rRNA gene libraries for each sample were quantified using QIAxpert (QIAGEN, Germany), pooled at the equimolar ratio, and used for pyrosequencing with the the MiSeq System (Illumina, USA) according to manufacturer's recommendations.

Taxonomic assignments by 16S rRNA genes sequence reads

Obtained raw pyrosequencing reads were filtered on the basis of the barcode and primer sequences using MiSeq Control Software version 1.1.1 (Illumina, USA). Sequence reads were taxonomically assigned using the MDx-Pro ver.1 profiling program (MD Healthcare Inc., Seoul, Korea). Briefly, the quality of sequence reads was retained by controlling an average PHRED score higher than 20

and read length of more than 300 bp. Operational taxonomic units (OTUs) were clustered using CD-HIT sequence clustering algorithms and were assigned using UCLUST [34] and QIIME [35] on the basis of the GreenGenes 8.15.13 16S rRNA sequence database [36]. Based on the sequence similarities, taxonomic assignments were achieved at the following levels: genus, $>94\%$ similarity; family, $>90\%$ similarity; order, $>85\%$ similarity; class, $>80\%$ similarity; and phylum, $>75\%$ similarity. In cases where clustering was not possible at the genus level due to a lack of sequence information at the database or redundant sequences, the taxon was named based on the higher-level taxonomy with parentheses.

Visualization and principal component analysis (PCA)

Data were normalized to have a mean of 0 and a standard deviation of 1 by linear normalization. PCA and two-dimensional scatter plots with axis of the first and second principal component were calculated and drawn using Matlab 2011a.

Statistical analysis

Two-sample comparisons were performed using Student's *t*-test. Data clustering of pyrosequencing reads were compared using the χ^2 test or *t*-test, while the comparisons between phylum compositions were tested by Fisher's exact test using GraphPad Prism 6 (San Diego, CA, USA). All data are presented as the mean \pm SEM, and a statistical difference was accepted at the 5% level.

RESULTS

Metagenome analysis of bodily microbiota in ASD individuals using bacteria-derived EVs in urine

Bacteria-derived EVs were isolated from the urine of 20 ASD individuals and 28 normal healthy subjects. The average age of the control and ASD subjects was 21.1 ± 9.5 years and 22.4 ± 4.9 years, respectively (Table 1). ASD subjects showed mild impairment of social interaction and stereotypes. The average K-CARS values of these ASD individuals was in the range between 31.5 and 33.5 and IQ values were in the range between 65 and 86. The control group was composed of healthy volunteers who had no medical problems including those related to ASD.

After the extraction of bacterial genomic DNA from the isolated EVs, variable regions of the 16S rRNA genes were amplified by PCR, and the libraries were constructed, as described previously [25, 26]. Subsequent DNA sequencing analyses led us to identify over 2,000 operational taxonomic units (OTUs) for ASD and normal individuals. There was no significant difference in the alpha diversity between the two groups (Fig. 1A). Among the identified OTUs, we assigned 30 OTUs at the phylum level, 75 OTUs at the

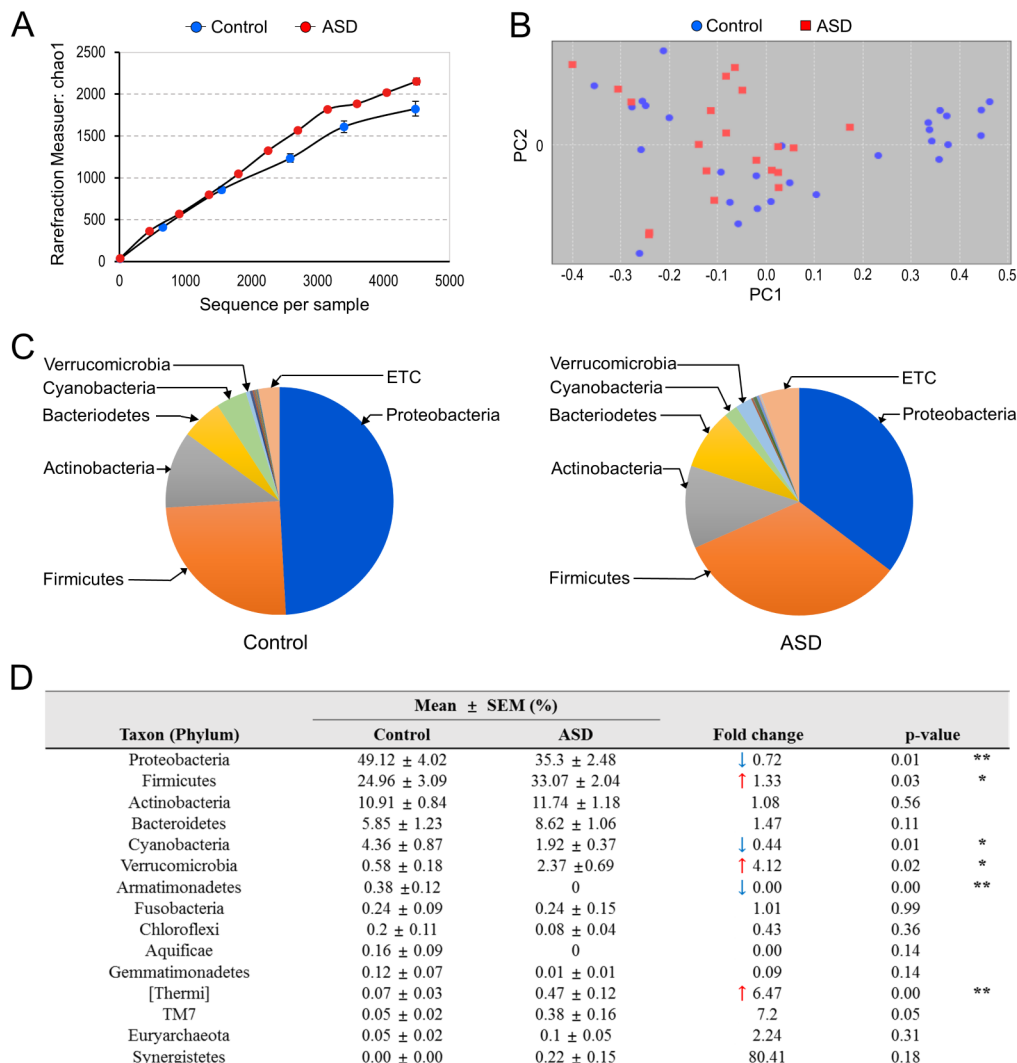


Fig. 1. The diversity and percent composition of microbiota at the phylum level in control vs. ASD subjects. (A) Rarefaction curves representing the mean OTUs over the identified sequences of variable regions of 16S rRNA gene in control (blue) and ASD (red) subjects. Data are the mean \pm SEM ($n=5$, each). (B) Principal component analysis of microbiota diversity based on the weighted UniFrac distance and Bray-Curtis dissimilarity. Data were normalized to have a mean of 0 and a standard deviation of 1. Control (blue) and ASD (red). (C) Overall composition of microbiota at the phylum level in control (blue) and ASD (red) subjects. Those with occupancy 0.1% or higher in control and/or ASD subjects are presented. (D) The percent composition of microbiota at the phylum level in control and ASD subjects. \uparrow and \downarrow denote an increase and decrease in the percent composition, respectively. Data are the mean \pm SEM ($n=5$, each). * and ** denote the differences between the indicated groups at $p<0.05$ and $p<0.01$, respectively (Student's t -test).

class level, 141 OTUs at the order level, 279 OTUs at the family level, and 619 OTUs at the genus level. Among these OTUs, we primarily focused on OTUs that occupied more than 0.1% of the identified taxa in the following analyses.

Altered microbiota profiles between ASD individuals and healthy subjects

Sequence readings of EVs-based 16S rDNA indicated that the top five members of the phyla *p*_Proteobacteria, *p*_Firmicutes, *p*_Actinobacteria, *p*_Bacteroidetes, and *p*_Cyanobacteria comprised 95.2% of the identified OTUs in healthy subjects, whereas these

members covered 90.65% of the total OTUs in ASD individuals, suggesting that ASD individuals have altered phyla composition. More specifically, the occupancy of *p*_Proteobacteria decreased from 49.12 to 35.30%, *p*_Cyanobacteria decreased from 4.36 to 1.92%, and *p*_Armatimonadetes decreased from 0.38 to 0.00% in ASD individuals (Fig. 1B-D). In contrast, the occupancy of *p*_Firmicutes increased from 24.96 to 33.07% and *p*_Verrucomicrobia increased from 0.58 to 2.37% in ASD.

The microbiota whose occupancy decreased or increased in ASD individuals were further analyzed at the class, order and family levels (Table 2, Supplemental Fig. S1). The decrease of *f*_

Table 2. The percent composition of microbiota at the class, order, and family taxonomic levels in control and ASD subjects

Taxon	Class			Order			Family						
	Mean (%)		Fold change	Mean (%)		Fold change	Mean (%)		Fold change				
	Control	ASD	p-value	Control	ASD	p-value	Control	ASD	p-value				
Gammaproteobacteria	23.51	23.47	1	0.99	0.21	2.62	12.68	0.02*	0.2	2.61	13.16	0.02*	
	15.29	4.84	0.32	0**	6.29	1.98	0.31	0**	6.27	1.98	0.32	0**	
					Rhizobiales	5.69	1.05	0.19	0**	4.58	0.18	0.04	0**
Betaproteobacteria	10.09	6.26	0.62	0.1	0.94	0.1	0.11	0.04*	0.46	0.08	0.17	0.01*	
					Burkholderiales	9.5	5.73	0.6	0.11	0.84	0.1	0.12	0.02**
									2.55	4.37	1.71	0.01*	
Deltaproteobacteria	0.2	0.64	3.23	0.01*	0.06	0.53	9.66	0**	0.06	0.53	9.66	0**	
	12.84	17.55	1.37	0.07	7.92	11.94	1.51	0.07	1.93	4.88	2.52	0.04*	
	11.63	15.4	1.32	0.18	11.59	15.38	1.33	0.18	1.06	1.87	1.75	0.04*	
Actinobacteria	9.9	11.09	1.12	0.38	7.84	10.29	1.31	0.09	0.44	1.19	2.70	0.02*	
					Actinomycetales				0	0.15	146.23	0.04*	
									0.4	1.56	3.91	0.02*	
Bacteroidia	5.05	7.28	1.44	0.21	2.06	0.8	0.39	0.03*	2.06	0.8	0.39	0.03*	
	0.56	1.19	2.11	0.03*	5.05	7.28	1.44	0.21	0.84	2.02	2.40	0.04*	
	3.81	1.76	0.46	0.03*	0.56	1.19	2.11	0.03*	0.43	1.12	2.63	0.01*	
Verrucomicrobiae	0.52	2.35	4.49	0.02*	3.8	1.68	0.44	0.03*	3.8	1.68	0.44	0.03*	
	0.38	0	0	0**	0.52	2.35	4.49	0.02*	0.52	2.35	4.49	0.02*	
	0.07	0.47	6.47	0**	0.38	0	0	0**	0.38	0	0.00	0**	
TM7-3	0.05	0.37	6.96	0.05*	0.03	0.21	6.3	0.02*	0.03	0.21	6.30	0.02*	

Microbiota at the family level whose occupancy was significantly different in ASD subjects are presented with associated higher taxonomy levels. Those occupying 0.1% or higher in either normal healthy or ASD subjects were included. ↑ and ↓ denote an increase and decrease in the percent composition, respectively. Data are the mean percentages. * and ** denote significant differences between the indicated groups at $p < 0.05$ and $p < 0.01$, respectively (Student's *t*-test).

Sphingomonadaceae and *f_Rhizobiaceae* accounted for the major decrease in *p_Proteobacteria* in ASD. In *p_Cyanobacteria*, *o_Streptophyta* decreased from 3.8 to 1.68%. In *p_Verrucomicrobia*, *f_Verrucomicrobiaceae* was the major increase (0.52 to 2.35%). In *p_Firmicutes*, *f_Streptococcaceae*, *f_Clostridiaceae*, an unclassified member of *o_Clostridiales* and *f_Eubacteriaceae* increased from 3.43 to 8.09% (Table 2).

The members of the genus occupied by more than 0.1% in either control or ASD individuals are summarized in Table 3 and Supplemental Table 1. Overall, 14 members at the genus level were down-regulated in ASD and their total occupancy in ASD dropped from 34.77 to 14.06%. On the contrary, 17 genus members were up-regulated in ASD and their total occupancy in ASD increased from 6.47 to 22.58%.

More specifically, an unclassified member of *f_Enterobacteriaceae* decreased from 8.85 to 6.08%, *g_Pseudomonas* decreased from 7.48 to 5.10%, *g_Sphingomonas* decreased from 4.17 to 0.71%, *g_Agrobacterium* decreased from 3.83 to 0.11%, an unclassified member of *o_Streptophyta* decreased from 3.80 to 1.68%, *g_Achromobacter* decreased from 2.42 to 0.05%, *g_Roseateles* decreased from 1.12 to 0.02%, and an unclassified member of *f_mitochondria* decreased from 0.84 to 0.10% (Table 3, Fig. 2).

On the other hand, *g_Streptococcus* increased from 1.58 to 4.77%, an unclassified member of *o_Clostridiales* increased from 1.06 to 1.87%, an unclassified member of *f_Comamonadaceae* increased from 0.92 to 3.79%, an unclassified member of *f_S24-7* increased from 0.84 to 2.02%, *g_Akkermansia* increased from 0.52 to 2.35%, *g_Rhodococcus* increased from 0.40% to 1.56%, and *g*

Table 3. The percent composition of microbiota at the genus level in control and ASD subjects

Class	Order	Family	Taxon	Mean±SEM (%)		Fold change	p-value			
				Control	ASD					
Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	0.12±0.06	1.72±0.51	↑ 14.61	0.01*			
		Pseudomonadaceae	Pseudomonas	7.48±0.86	5.10±0.6	↓ 0.68	0.03*			
	Enterobacteriales	Enterobacteriaceae	Erwinia	0.26±0.1	0.64±0.15	↑ 2.41	0.04*			
			Citrobacter	0.66±0.24	0.08±0.05	↓ 0.12	0.02*			
			Unclassified	8.85±1.01	6.08±0.58	↓ 0.69	0.02*			
Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	4.17±0.83	0.71±0.2	↓ 0.17	0.00**			
		Rhizobiales	Rhizobiaceae	Agrobacterium	3.83±0.96	0.11±0.05	↓ 0.03	0.00**		
				Unclassified 1	0.63±0.17	0.07±0.03	↓ 0.11	0.00**		
				Unclassified 2	0.11±0.03	0.00±0.00	↓ 0.00	0.00*		
				Unclassified	0.24±0.07	0.05±0.02	↓ 0.21	0.02*		
				Rickettsiales	Mitochondria	Unclassified	0.84±0.3	0.1±0.06	↓ 0.12	0.02*
				Rhodobacterales	Rhodobacteraceae	Rhodobacter	0.01±0.01	0.23±0.08	↑ 20.34	0.02*
Betaproteobacteria	Burkholderiales	Comamonadaceae	Roseateles	1.12±0.35	0.02±0.01	↓ 0.02	0.00**			
			Delftia	0.22±0.08	0.01±0.00	↓ 0.05	0.02*			
			Comamonas	0.08±0.05	0.36±0.12	↑ 4.48	0.04*			
			Unclassified	0.92±0.15	3.79±0.49	↑ 4.14	0.00**			
			Alcaligenaceae	Achromobacter	2.42±0.75	0.05±0.03	↓ 0.02	0.00**		
			Desulfobacteriales	Desulfobacteriaceae	Desulfobacterium	0.04±0.02	0.48±0.14	↑ 10.88	0.00*	
Deltaproteobacteria	Desulfobacteriales	Desulfobacteriaceae	Desulfobacterium	0.04±0.02	0.48±0.14	↑ 10.88	0.00*			
			Desulfobacterium	0.04±0.02	0.48±0.14	↑ 10.88	0.00*			
Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	1.58±0.26	4.77±1.28	↑ 3.03	0.02*			
	Bacillales	Staphylococcaceae	Jeotgalicoccus	0.03±0.02	0.50±0.11	↑ 14.70	0.00*			
Clostridia	Clostridiales	Unclassified	Unclassified	1.06±0.26	1.87±0.26	↑ 1.75	0.04*			
		Clostridiaceae	Unclassified	0.27±0.08	0.70±0.18	↑ 2.58	0.04*			
		Ruminococcaceae	Oscillospira	0.10±0.04	0.47±0.14	↑ 4.53	0.02*			
Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	0.40±0.13	1.56±0.43	↑ 3.91	0.02*			
		Micrococcaceae	Kocuria	0.06±0.05	0.30±0.08	↑ 4.75	0.01*			
Bacteroidia	Bacteroidales	S24-7	Unclassified	0.84±0.36	2.02±0.44	↑ 2.40	0.04*			
Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Cloacibacterium	0.13±0.06	0.62±0.2	↑ 4.92	0.03*			
Chloroplast	Streptophyta	Unclassified	Unclassified	3.80±0.84	1.68±0.38	↓ 0.44	0.03*			
Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	0.52±0.18	2.35±0.68	↑ 4.52	0.02*			
[Fimbriimonadia]	[Fimbriimonadales]	[Fimbriimonadaceae]	Fimbriimonas	0.38±0.12	0.00±0.00	↓ 0.00	0.00**			
Deinococci	Thermales	Thermaceae	Thermus	0.03±0.02	0.21±0.07	↑ 6.30	0.02*			

Microbiota at the genus level whose occupancy was significantly different in ASD subjects are presented with associated higher taxonomy levels. Microbiota with occupancy 0.1% or higher in either normal healthy or ASD subjects were considered. ↑ and ↓ denote an increase and decrease in the percent composition, respectively.

Data are the mean percentage±SEM. * and ** denote significant differences between the indicated groups at p<0.05 and p<0.01, respectively (Student's *t*-test).

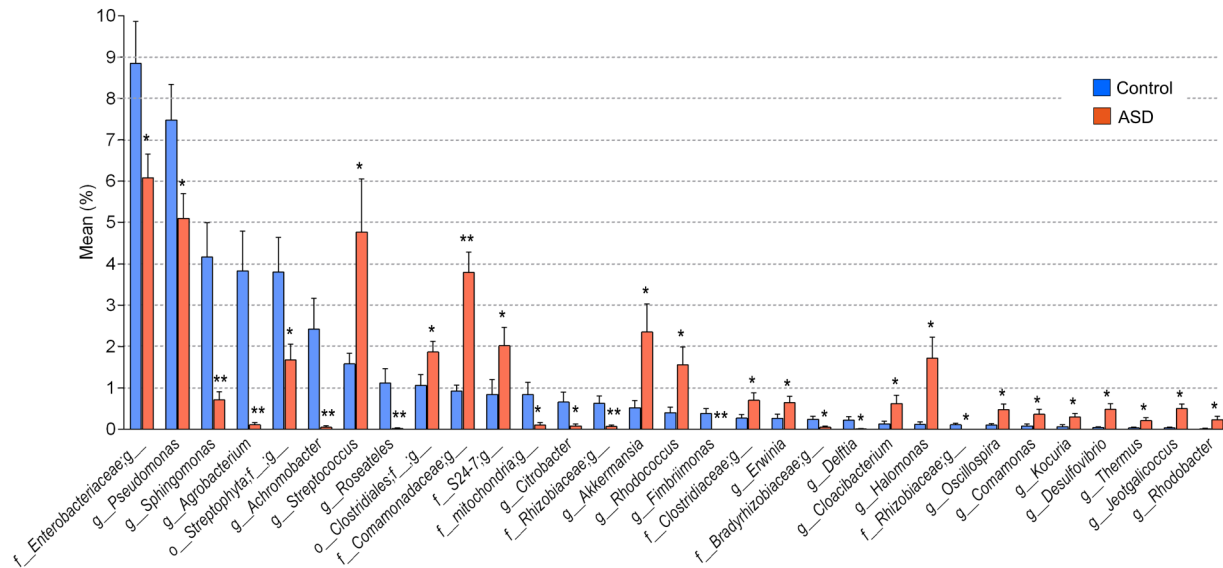


Fig. 2. Genus members that were downregulated or upregulated in ASD subjects. The percent composition of microbiota whose occupancy was significantly changed at the genus level among those occupying 0.1% or higher in either control (blue) or ASD (red) subjects. Data are presented as the mean percentage±SEM. * and ** denote the differences between the indicated groups at p<0.05 and p<0.01, respectively (Student's *t*-test).

Halomonas increased from 0.12 to 1.72% (Table 3, Fig. 2).

DISCUSSION

Metagenome analysis of bacterial EVs in urine identifies altered microbiota profiles in ASD

In the present study, we demonstrated that bacteria-derived EVs in urine were useful for the rapid assessment of microbiota profiles in ASD. The metagenome analysis of urine EVs indicated that *p_Verrucomicrobia* (0.58 to 2.37%, p=0.02) and *p_Firmicutes* (24.96 to 33.07%, p=0.03) increased in ASD, whereas *p_Cyanobacteria* (4.36 to 1.92%, p=0.01) and *p_Proteobacterium* (49.12 to 35.3%, p=0.01) decreased. There was no significant change in *p_Bacteroidetes* (5.85 to 8.62%, p=0.11) and *p_Actinobacterium* (10.91 to 11.74%, p=0.56). The altered microbiota compositions identified from urine EVs of ASD were partially consistent with microbiota compositions assessed from fecal samples reported in recent studies. The analyses of fecal microbiota compositions in previous studies reported that *p_Actinobacteria*, *p_Verrucomicrobia* and *p_Cyanobacteria* decreased or tended to decrease in ASD, but there were conflicting results for *p_Firmicutes*, *p_Bacteroidetes* and *p_Proteobacteria* (Table 4) [13, 15, 16, 19].

In *p_Firmicutes*, *g_Streptococcus* (1.58 to 4.77%, p=0.02), *g_Jeotgalicoccus* (0.03 to 0.5%, p<0.01), *g_Oscillospira* (0.1 to 0.47%, p=0.02), and an unclassified member of *f_Clostridiaceae* (0.27 to 0.7%, p=0.04) significantly increased in ASD by more than two-fold. The genera *g_Akkermansia* occupied the greatest propor-

tion of the increased phylum *p_Verrucomicrobia* (0.52 to 2.35%, p=0.02), whereas in *p_Proteobacterium*, 12 genera decreased and 5 genera increased in ASD. The genera *g_Pseudomonas*, *g_Citrobacter*, an unclassified member of *f_Enterobacteriaceae*, *g_Sphingomonas*, *g_Agrobacterium*, unclassified members of *f_Rhizobiaceae*, an unclassified member of *f_Bradyrhizobiaceae*, and an unclassified member of *f_mitochondria*, *g_Roseateles*, *g_Delftia* and *g_Achromobacter* were decreased (total 30.58 to 12.37%), whereas *g_Halomonas*, *g_Erwinia*, *g_Rhodobacter*, *g_Comamonas*, and an unclassified member of *f_Comamonadaceae* were increased (total 1.39 to 6.74%).

At the genus level, the present study identified *g_Sphingomonas*, *g_Agrobacterium*, an unclassified member of *o_Streptophyta*, *g_Achromobacter*, and *g_Roseateles* as being decreased in ASD by more than two-fold among the total OTUs having occupancy 0.1% or higher in either healthy control or ASD subjects, and *g_Streptococcus*, an unclassified member of *f_Comamonadaceae*, an unclassified member of *f_S24-7*, *g_Akkermansia*, *g_Rhodococcus*, and *g_Halomonas* were increased in ASD. Particularly, *g_Desulfovibrio* increased in ASD (0.04 to 0.48%, p<0.01), *g_Lactobacillus* tended to increase (2.56 to 5.45%, p=0.08), *g_Bifidobacterium* tended to decrease (1.9 to 0.8%, p=0.06), and *g_Turicibacter* tended to decrease (0.19 to 0.02%, p=0.07) in ASD, whereas *g_Enterococcus* (0.4 to 0.78%, p=0.06), *g_Enterobacter* (0.03 to 0.37, p=0.08), and *g_Clostridium* (0.11 to 0.31%, p=0.09) tended to increase in ASD. The changes in these genus members are broadly consistent with the results of previous reports assessed

Table 4. Summary and comparison of microbiota characterized in the present study with those identified from fecal samples in previous studies

	Taxons	Mean (%)		Fold change	p-value	Literatures
		Control	ASD			
Phylum	Proteobacteria	49.12	35.30	↓ 0.72	0.01**	↓ [19]; ↑ [13]
	Firmicutes	24.96	33.07	↑ 1.33	0.03*	↓ [13]; ↑ [15]
	Actinobacteria	10.91	11.74	↑ 1.08	0.56	↓ [13]
	Bacteroidetes	5.85	8.62	↑ 1.47	0.11	↑ [13]; ↓ [15]; ↓ [19]
	Verrucomicrobia	0.58	2.37	↑ 4.12	0.02*	↓ [19]
Class	Betaproteobacteria	10.09	6.26	↓ 0.62	0.10	↑ [15]
Order	Clostridiales	11.59	15.38	↑ 1.33	0.18	↑ [15]
Family	Ruminococcaceae	5.46	6.11	↑ 1.12	0.68	↑ [15]
	Lachnospiraceae	3.12	2.57	↓ 0.82	0.54	↑ [15]
	Corynebacterium	2.60	3.38	↑ 1.3	0.34	↑ [11]
	Alcaligenaceae	2.53	0.12	↓ 0.05	0.00**	↑ [16]
	Pseudomonas	7.48	5.10	↓ 0.68	0.03*	↑ [21]
	Lactobacillus	2.56	5.45	↑ 2.13	0.08	↑ [18]; ↑ [11]
	Bacteroides	2.48	2.93	↑ 1.18	0.65	↑ [13]; ↑ [21]
	Staphylococcus	2.23	2.57	↑ 1.15	0.66	↓ [21]
	Faecalibacterium	2.14	2.05	↓ 0.96	0.90	↑ [20]
	Bifidobacterium	1.90	0.80	↓ 0.42	0.06	↓ [13]; ↓ [18]; ↓ [21]; ↓ [17]
	Streptococcus	1.58	4.77	↑ 3.03	0.02*	↓ [13]; ↓ [21]
	Akkermansia	0.52	2.35	↑ 4.52	0.02*	↓ [19]; ↓ [17]
	Blautia	0.47	0.28	↓ 0.59	0.38	↓ [20]
	Enterococcus	0.40	0.78	↑ 1.92	0.06	↑ [21]; ↓ [18]
	Collinsella	0.37	0.11	↓ 0.29	0.16	↓ [13]; ↑ [11]
	Veillonella	0.37	0.50	↑ 1.34	0.43	↓ [11]
	Lactococcus	0.35	0.11	↓ 0.3	0.16	↓ [13]; ↓ [21]
	[Ruminococcus]	0.34	0.15	↓ 0.45	0.16	↓ [13]
	Coprococcus	0.28	0.27	↓ 0.94	0.90	↓ [19]
	Leuconostoc	0.27	0.01	↓ 0.02	0.14	↓ [13]
	Dialister	0.23	1.39	↑ 6.16	0.18	↓ [13]; ↓ [11]
	Parabacteroides	0.21	0.18	↓ 0.83	0.76	↓ [11]; ↑ [13]
	Weissella	0.21	0.07	↓ 0.31	0.33	↓ [13]
	Turicibacter	0.19	0.02	↓ 0.12	0.07	↓ [13]
	Dorea	0.17	0.10	↓ 0.6	0.44	↑ [11]
	Clostridium	0.11	0.31	↑ 2.72	0.09	↑ [21]; ↓ [13]
	[Prevotella]	0.09	0.12	↑ 1.34	0.74	↑ [21]; ↓ [19]
	Desulfovibrio	0.04	0.48	↑ 10.88	0.00**	↑ [13]; ↓ [19]
	Genus	0.03	0.37	↑ 11.53	0.08	↑ [21]

The microbiota whose percent composition were significantly different in ASD subjects as characterized in the present study were compared with those identified from fecal samples in previous studies. ↑ and ↓ denote an increase and decrease in the percent composition, respectively. * and ** denote significant differences between the indicated groups at $p < 0.05$ and $p < 0.01$, respectively (Student's t-test).

for fecal microbiota compositions (Table 4) [11, 13, 17, 18, 21].

The EV levels of *g_Oscillospira*, unclassified members of *f_Clostridiaceae* and *f_Eubacteriaceae*, and an unclassified member of *o_Clostridiales* were increased in ASD subjects. Previous studies have reported that several species of *c_Clostridia* produced 4-ethylphenyl sulfate (4-EPS) and *p*-cresol, which were found at high concentrations in the urine of ASD children. Administration of 4-EPS in healthy mice produced myelination deficits in the prefrontal cortex and sociability defects [37-39]. It was also reported that *c_Clostridia* produced propionic acid (PPA) and its related short-chain fatty acids (SCFAs) as fermentation products, and PPA

infusions in rats induced ASD-linked neurochemical and behavioral changes [40]. These results suggest that bacteria-derived metabolites induce neurochemical and structural changes and shape behavioural abnormalities.

Oral treatment with *Bifidobacteria fragilis* ameliorated ASD-related gastrointestinal deficits and associated behavioural abnormalities behavioral abnormalities in the poly (I:C)-injection model [41]. *Bifidobacteria infantis* attenuated pro-inflammatory immune responses and production of serotonergic precursor, tryptophan, and has potential anti-depressant properties [42, 43]. Considering these results, ASD groups with decreased EV levels

of *g_Bifidobacterium* might have benefits by probiotic treatment with *g_Bifidobacterium*.

Bacterial EVs in urine are useful for rapid assessment of bodily microbiota profiles in ASD

The microbiota profile assessed from urine EVs might reflect a large part of the gut microbiota. Nonetheless, we do believe that the microbiota profile assessed from urine EVs is not likely a simple alternative for microbiota profile assessed from stool. Possible sources for metagenome analysis of bodily microbiota may include stool bacteria, stool EVs, gut (ex, stomach and/or specific regions of the small and large intestines) bacteria, respiratory exhale EVs, oral/nasal bacteria and EVs, urinary system bacteria and EVs, and blood EVs. Generally speaking, microbiota in stool represents the intestinal compartment, whereas microbiota in urine or blood reflects the whole body including the intestinal compartments, oral system, respiratory system, and urinary system. Nonetheless, among the body parts, the gut is the major source of bodily microbiota. It was reported that the metabolites of intestinal microbiota activities, including phenyllactate, p-cresol sulfate, concentrations, and serotonin in urine, plasma, and stool of mouse pups undernourished by timed separation from lactating dams, then resumed ad libitum nursing, were different from each other, although they had some correlations [44, 45]. Similar to metabolite profiles of intestinal microbiota activities, available information suggests that metagenome analysis assessed from these sources might be closely related, but represent some distinct landscapes. For an example, metagenome analysis of bacteria and bacteria-derived EV in stool of inflammatory bowel disease model mice indicated that the EV composition in stool was more drastically altered compared to that of bacterial composition in stool [25]. Considering that bacteria-derived EV indicates the metabolically or pathologically activated microbiota [25, 27], urine EV may be more representative of the host's microbiota activities than stool bacteria.

To the best of our knowledge, this is the first report characterizing microbiota in ASD individuals on the basis of urine EVs. Compared to blood and feces, urine is easily obtained in large volume and is readily available via a non-invasive method. Considering the general difficulties in repeated sampling microbiota sources from ASD individuals, particularly low functioning individuals with ASD or toddlers with ASD, using urine as a sample source would be a great advantage for rapid and repeated assessments of microbiota changes under varying physiological contexts compared to the use of blood and feces. Comparative analyses of EV profiles from urine, blood and stool of ASD individuals will be valuable. Also it will be worth to understand EV profiles of ASD with diverse factors including age, sex, familial history, genetics,

and ethnics.

Overall, the present study assessed urine EVs from individuals with mildly autistic subjects. We believe that further systematic and unbiased analyses of male and female subjects with broad ASD spectrums are necessary. This study focused on young adult subjects. Considering that ASD should be diagnosed in young children as early as 1.5~3 yr of age, this analysis should be expanded to toddlers and infants.

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