

## Original Article



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# Diagnostic Models for Atopic Dermatitis Based on Serum Microbial Extracellular Vesicle Metagenomic Analysis: A Pilot Study

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## ABSTRACT


**Purpose:** Associations between a wide variety of diseases and the microbiome have been extensively verified. Recently, there has been a rising interest in the role the microbiome plays in atopic dermatitis (AD). Furthermore, metagenomic analysis of microbe-derived extracellular vesicles (EVs) has revealed the importance and relevance of microbial EVs in human health.

**Methods:** We compared the diversity and proportion of microbial EVs in the sera of 24 AD patients and 49 healthy controls, and developed a diagnostic model. After separating microbial EVs from serum, we specifically targeted the V3–V4 hypervariable regions of the *16S rDNA* gene for amplification and subsequent sequencing.

**Results:** Alpha and beta diversity between controls and AD patients both differed, but only the difference in beta diversity was significant. Proteobacteria, Firmicutes, and Bacteroidetes were the dominant phyla in healthy controls and AD patients, accounting for over 85% of the total serum bacterial EVs. Also, Proteobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, and Cyanobacteria relative abundances were significantly different between the AD and control groups. At the genus level, the proportions of *Escherichia-Shigella*, *Acinetobacter*, *Pseudomonas*, and *Enterococcus* were drastically altered between the AD and control groups. AD diagnostic models developed using biomarkers selected on the basis of linear discriminant analysis effect size from the class to genus levels all yielded area under the receiver operating characteristic curve, sensitivity, specificity, and accuracy of value 1.00.

**Conclusions:** In summary, microbial EVs demonstrated the potential in their use as novel biomarkers for AD diagnosis. Therefore, future work should investigate larger case and control groups with cross-sectional or longitudinal clinical data to explore the utility and validity of serum microbiota EV-based AD diagnosis.

**Keywords:** Microbiome; extracellular vesicles; metagenomics; biomarkers; atopic dermatitis; IgG

Young-Koo Jee <https://orcid.org/0000-0001-5800-8038>Hae-Sim Park <https://orcid.org/0000-0003-2614-0303>Bok Yang Pyun <https://orcid.org/0000-0002-6375-9394>**Disclosure**

There are no financial or other issues that might lead to conflict of interest.

## INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disorder and its evolution is characterized by the interaction between several genetic risk factors and environmental triggers.<sup>1,2</sup> Numerous genetic risk factors for AD have been confirmed with an increased emphasis on the importance of innate and adaptive immune pathways and skin barrier function.<sup>3</sup> Filaggrin (FLG) is a key protein that is involved in epidermal terminal differentiation and skin barrier function, and loss-of-function mutations in the *FLG* gene are considered the greatest risk factor for AD.<sup>4</sup> FLG is also highly related to immunoglobulin E (IgE)-mediated allergen sensitization, and approximately 42% of AD patients have been shown to possess a FLG mutation.<sup>5,6</sup> The major causes of AD are skin barrier dysfunction, allergic sensitization, microbial skin colonization, and reduced innate immune responses.<sup>7,8</sup>

While AD onset usually occurs during childhood and lasts throughout adulthood, adulthood onset of AD can occur as well.<sup>9</sup> With a lifetime prevalence rate of 20%, approximately 2 million children globally suffer from AD. Recently, incidence rates of AD among children are increasing in affluent countries and showing disproportionate rates between developing and developed countries.<sup>10</sup> Aside from physical discomfort, AD also causes children to suffer from extreme psychological stress. According to a report, 84% of children with AD have a hard time falling asleep at night and more than one-third are more sensitive about their appearance.<sup>11</sup>

Extensive research of the human microbiota, the total microbial community residing in our bodies, has provided a preliminary understanding of the biology and medical significance of the human microbiome and its collective genes.<sup>12</sup> Additionally, associations between various diseases and the microbiome have been verified. Furthermore, studies of the extracellular vesicle (EV) metagenome have proven to be advantageous as it provides an excellent tool to probe the effects of bacterial infection at the systematic level.<sup>13</sup> EVs are nanometer-sized particles that can be isolated from body fluids including serum, plasma, and urine. They function not only as intercellular signaling mediators using enclosed proteins, nucleic acids, metabolites, and lipids, but serve as a source of novel biomarkers in disease diagnosis to assess whether or not an individual has a disease, while risk assessment is the prediction of whether an individual has the potential to develop a disease or if they are at risk of developing a more severe or less severe case of a disease.<sup>13,15</sup>

Recently, there has been an increased research interest in the role of the microbiome in AD.<sup>16</sup> The results of these studies have shown that there are significant differences in microbial diversity and abundance between AD patients and healthy individuals.<sup>16-18</sup> However, the target of these studies was bacteria in the skin microbiome, but not microbial EVs in the human body. Since the skin is directly affected by the atmosphere, the samples are more likely to be contaminated by the environment during sampling than those collected from inside the body. Here, we analyzed the diversity and abundance of microbial EVs in sera collected from AD patients and healthy controls through 16S rDNA metagenomic analysis using next-generation sequencing (NGS). We then developed diagnosis models based on multiple regression analysis of biomarkers selected through the analysis of biologically and statistically significant bacterial EV taxa as a pilot study.

## MATERIALS AND METHODS

### Subjects and serum sample collection

In total, 24 AD patients (15 males and 9 females) and 49 controls (35 male and 14 female) were enrolled from Soonchunhyang University Hospital from 2015 to 2016. AD participants were recruited from Soonchunhyang University Hospital and sample collection was conducted after obtaining consent from the patients and their parents. Each of the clinical subjects showed very severe AD symptoms causing them to visit the hospital for treatment. Children with moderate to severe AD diagnosed according to Hanifin and Rajka's diagnostic criteria by pediatric allergy specialists were included in the study.<sup>19</sup> The severity of AD was evaluated using the SCORAD index<sup>20</sup> and disease duration (over 6 months). Healthy control subjects were screened through a general health examination. The present study was approved by the Institutional Review Board of Soonchunhyang University Hospital (IRB No. SCHUH 2013-06-001). The methods conducted in this study were in accordance with the approved guidelines, and informed consent was obtained from each subject.

### EV isolation and DNA extraction from human serum samples

The serum sample was transferred to a Serum Separator Tube (Becton Dickinson, USA) and then centrifuged at 3,000 rpm for 15 minute at 4°C. To isolate EVs from serum samples, after the resulting supernatant was diluted in 1× PBS, floating particles were pelleted by centrifugation at 10,000× *g* for 1 minute at 4°C. The supernatant containing bacterial EVs was collected. Next, bacteria and foreign particles in the supernatant were eliminated through filtering by 0.22-µm filters. To extract DNA, the EVs obtained were boiled for 40 minutes at 100 °C and centrifuged for 30 minutes at 13,000 rpm at 4°C. EV DNA was then extracted using a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). Finally, the EVs DNA in each sample was quantified using QIAxpert system (QIAGEN).

### 16S rDNA amplicon sequencing using EV DNA from human serum samples

V3-V4 hypervariable regions of the *16S rDNA* gene of EVs DNA obtained were amplified with 16S\_V3\_F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 16S\_V4\_R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAA TCC-3') primers. The libraries were prepared by PCR products according to the MiSeq System guide (Illumina, San Diego, CA, USA) and quantified using the QIAxpert system (QIAGEN). Each amplicon was sequenced on a MiSeq (Illumina).

### Analysis of bacterial composition in the microbiome

Paired-end reads were trimmed by cutadapt (ver. 1.1.6). The resulting FASTQ files containing paired-end reads were merged using CASPER and then quality filtered by Phred (Q) score. After merging any reads under 350 bp or over 550 bp were also discarded. Next, a reference-based chimera detection step was performed using VSEARCH against the SILVA gold database. The sequence reads were clustered into operational taxonomic units (OTUs) using *De novo* clustering algorithm and the threshold was 97% sequence similarity. Finally, OTUs were classified using UCLUST under default parameters with SILVA 128 database.

### Statistical analysis

Significant differences between AD and control groups were tested using a  $\chi^2$  test for categorical variables, and a *t* test and a Wilcoxon rank-sum test for continuous variables. Student's *t* tests were additionally performed to compare the relative abundance of microbial taxa between AD and control groups. A *P* value less than 0.05 was considered statistically

significant. To discover biomarkers, the linear discriminant analysis (LDA) effect size (LEfSe) considering statistical significance and biological relevance was performed. The criteria for serum bacterial EV biomarker selection were to occupy an average relative abundance greater than 1% in any group, have a *P* value lower than 0.05 by a Kruskal-Wallis rank-sum test between control and AD patients, and have an LDA score greater than 3. Logistic regression using biomarkers that met the above criteria was performed to develop AD diagnostic models. All statistical analyses were performed using R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/index.html>).

## RESULTS

### Demographic characteristics of the study subjects

A total of 73 subjects were enrolled that included 24 AD patients and 49 healthy controls. The difference in sex between healthy control and patients group was not significant (*P* = 0.71). The female age differences in the AD and healthy control groups were significant (*P* = 0.04), but the male ages were not significantly different (*P* = 0.10) (Table).

### Comparison of serum microbial EV composition diversity between clinical groups

In order to perform microbiome analysis, 16S rDNA was sequenced from purified DNA extracted from microbial EVs obtained from serum collected from healthy controls and AD patients. To clarify the alpha diversity of the serum microbial EV composition of AD and healthy controls, the Chao1 index was used to estimate microbial richness and then expressed as a rarefaction curve. The slope of the rarefaction curve was shown to be steeper in the healthy control group than in the AD group, indicating higher alpha diversity in the control group than in the AD group (Fig. 1A). The microbial community diversity estimated by the Shannon diversity index did not significantly differ (*P* = 0.095) (Fig. 1B), and the OTUs of the healthy controls appeared to be approximately 4.5 times greater than those of the AD patients. The mean  $\pm$  standard deviation valid reads were 46,020.1  $\pm$  29,015.8 in healthy controls and 9,006.1  $\pm$  3,434.4 in AD patients.

The differences in the serum microbial EV composition between healthy controls and AD patients were evaluated through principal coordinate analysis (PCoA). These 2 clinical groups were significantly divided from principal coordinate 1 (PCo1) and principal coordinate 2 (PCo2) (*p* < 0.001) at the phylum, class, order, family, and genus levels (Fig. 2).

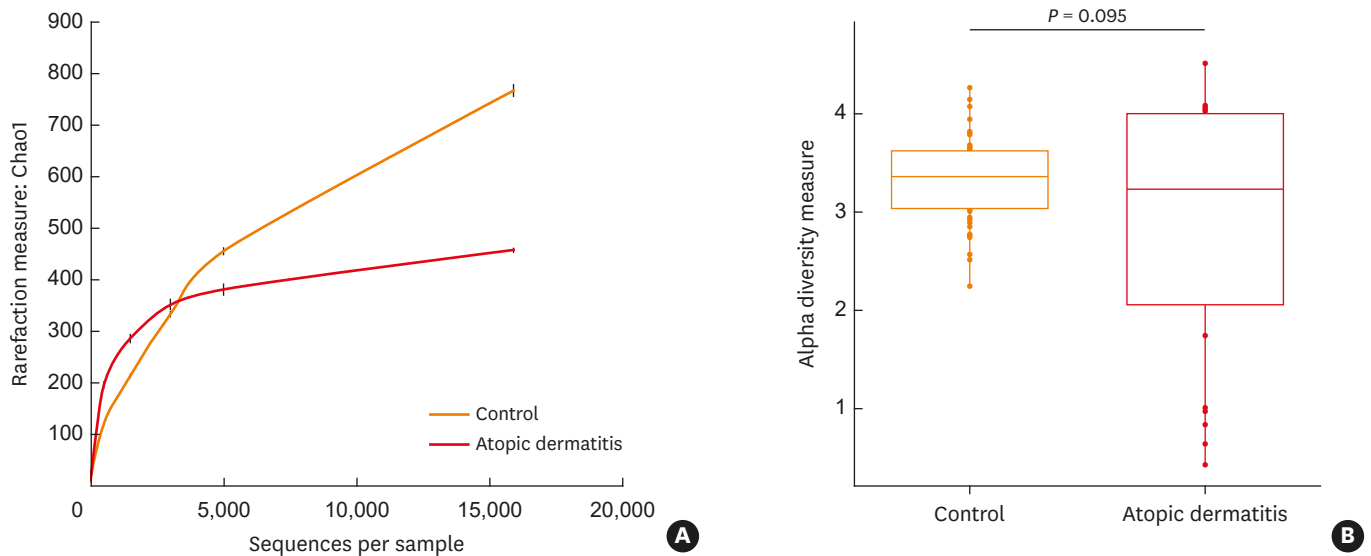
### Microbial distribution throughout blood

At the phylum level, there were 9 bacterial taxa over 0.1% in the healthy controls and 6 in the AD patients. Proteobacteria, Firmicutes and Bacteroidetes were dominant in the healthy

**Table.** Basic clinical subject characteristics

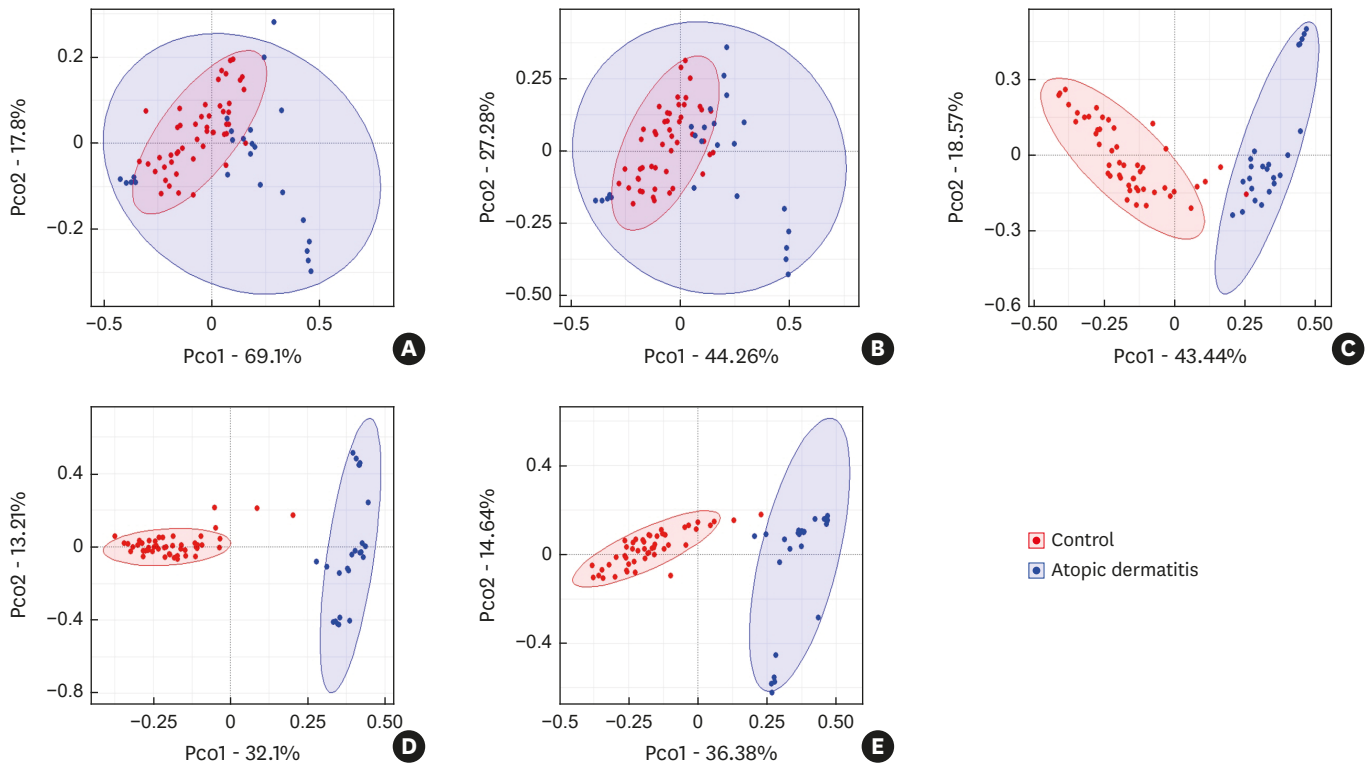
Variables	Control (n = 49)	AD (n = 24)	<i>P</i> value
Sex (Male/Female)	35/14	15/9	0.92
Age* (years)			
Total	7.4 $\pm$ 5.3	10.5 $\pm$ 1.5	0.01
Male	7.9 $\pm$ 5.6	10.5 $\pm$ 1.5	0.10
Female	6.4 $\pm$ 5.1	10.6 $\pm$ 1.7	0.04
SCORAD index*	-	71.8 $\pm$ 18.1	-

Data are expressed as mean  $\pm$  standard deviation not otherwise specified. AD, atopic dermatitis.



**Fig. 1.** Alpha diversity of the control and AD groups. (A) Alpha-diversity rarefaction curves based on the Chao1 index to estimate richness. (B) The Shannon index was used to estimate serum bacterial EV community richness and evenness. AD, atopic dermatitis; EV, extracellular vesicle.

controls and the AD patients, accounting for over 85% of bacterial abundance. Actinobacteria followed with 5.7% abundance in healthy controls and 9.5% in AD patients. Proteobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, and Cyanobacteria differed significantly between the healthy controls and the AD patients ( $P < 0.05$ ). The amounts of Proteobacteria



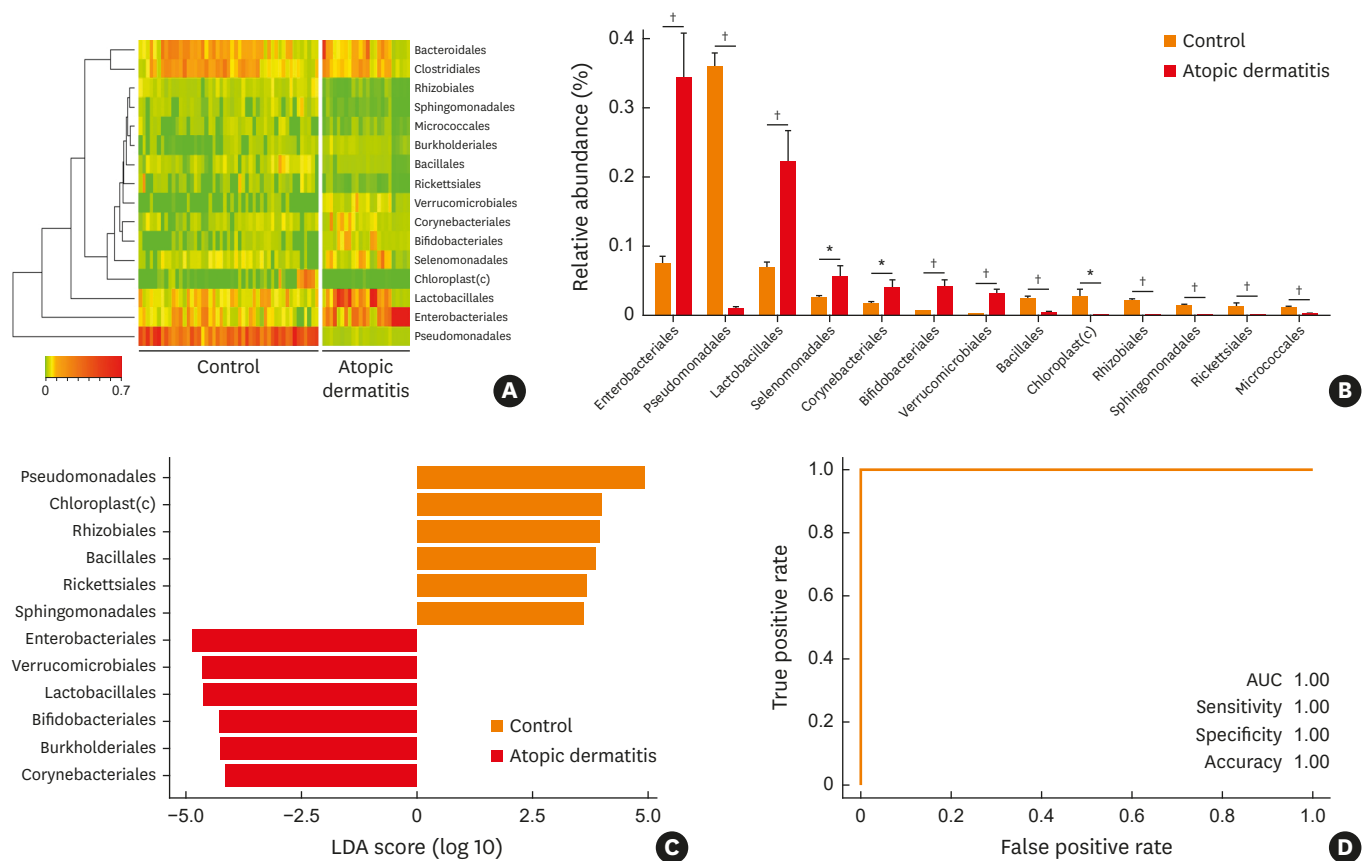
**Fig. 2.** PCoA of control and AD group serum microbial EVs at the (A) phylum, (B) class, (C) order, (D) family, and (E) genus levels. PCoA, principal coordinate analysis; AD, atopic dermatitis; EV, extracellular vesicle.

(52.8%–37.8%) and Cyanobacteria (3.3%–0.1%) were shown to decrease in AD patients, while the amounts of Verrucomicrobia (0.3%–3.2%), Actinobacteria (5.7%–9.5%) and Firmicutes (24.5%–38.3%) were shown to increase (**Supplementary Fig. S1A and B**). Four key phylum-level biomarkers were selected that met the following phyla which: accounted for over 1% abundance in any group, had significant difference ( $P < 0.05$ ) between the control and AD groups, and yielded LDA scores greater than 3 based on LEfSe assessment. Of these 4 phylum-level biomarkers, Proteobacteria and Cyanobacteria were increased in the control group, whereas Firmicutes and Verrucomicrobia were decreased (**Supplementary Fig. S1C**). Internal model validation showed that the area under the receiver operating characteristic (ROC) curve (AUC), sensitivity, specificity, and accuracy were 0.99, 1.00, 0.96, and 0.97, respectively (**Supplementary Fig. S1D**).

At the class level, 17 and 13 bacterial taxa occupied over 0.1% of the total bacterial population in the healthy control subjects and the AD patients, respectively. Gammaproteobacteria, Bacilli, Bacteroidia, and Clostridia were dominant in both healthy controls and AD patients, accounting for over 75% of bacterial abundance. Actinobacteria followed with 5.2% abundance in the healthy controls, and 8.8% in the AD patients. The amounts of Alphaproteobacteria (5.8%–0.6%) and Chloroplast (3.2%–0.1%) were decreased in AD patients, while the amounts of Bacilli (9.4%–22.7%), Actinobacteria (5.2%–8.8%), Negativicutes (2.5%–5.7%), and Verrucomicrobiae (0.3%–3.2%) were increased (**Supplementary Fig. S2A and B**). LEfSe analysis revealed 5 class-level biomarkers that comprised Gammaproteobacteria, Alphaproteobacteria and Chloroplast, which were increased in the healthy controls, and Bacilli and Verrucomicrobiae, which were decreased in the control group (**Supplementary Fig. S2C**). The results of the model internal validation showed that sensitivity, specificity, accuracy and AUC were all expressed as 1.00 (**Supplementary Fig. S2D**).

Meanwhile, 34 and 22 bacterial orders accounted for over 0.1% of the total bacterial abundance in the healthy controls and the AD patients, respectively. Pseudomonadales was dominant in the healthy controls with 36.0% abundance, while Enterobacteriales was dominant in the AD patients with 34.4% abundance. Actinobacteria were the next most abundant order accounting for 5.2% in the healthy controls and 8.8% in the AD patients. The amounts of Pseudomonadales (36.0%–1.0%), Bacillales (2.4%–0.4%), Chloroplast(c) (2.8%–0.0%), Rhizobiales (2.1%–0.1%), Sphingomonadales (1.4%–0.1%), Rickettsiales (1.3%–0.1%) and Micrococcales (1.1%–0.3%) were shown to decrease in the AD patients, while the amounts of Enterobacteriales (7.6%–34.4%), Lactobacillales (7.0%–22.2%), Selenomonadales (2.5%–5.7%), Bifidobacteriales (0.6%–4.2%), and Verrucomicrobiales (0.3%–3.2%) were shown to increase (**Fig. 3A and B**). Based on LEfSe analysis, the key order-level biomarkers that were increased in the healthy controls were Pseudomonadales, Chloroplast(c), Rhizobiales, Bacillales, Rickettsiales and Sphingomonadales; whereas in the control group, Enterobacteriales, Verrucomicrobiales, Lactobacillales, Bifidobacteriales, Burkholderiales, and Corynebacteriales were decreased (**Fig. 3C**). Model sensitivity, specificity, accuracy, and AUC were all revealed to be 1.00, through internal evaluation of the resulting diagnostic model (**Fig. 3D**).

At the family level, there were 52 bacterial taxa over 0.1% in the healthy controls and 34 in the AD patients. Enterobacteriaceae and Enterococcaceae were dominant in the AD patients with 34.4% and 12.6% abundance, respectively, while Moraxellaceae and Pseudomonadaceae were dominant in the healthy controls with 22.6% and 13.4% abundance, respectively. The portions



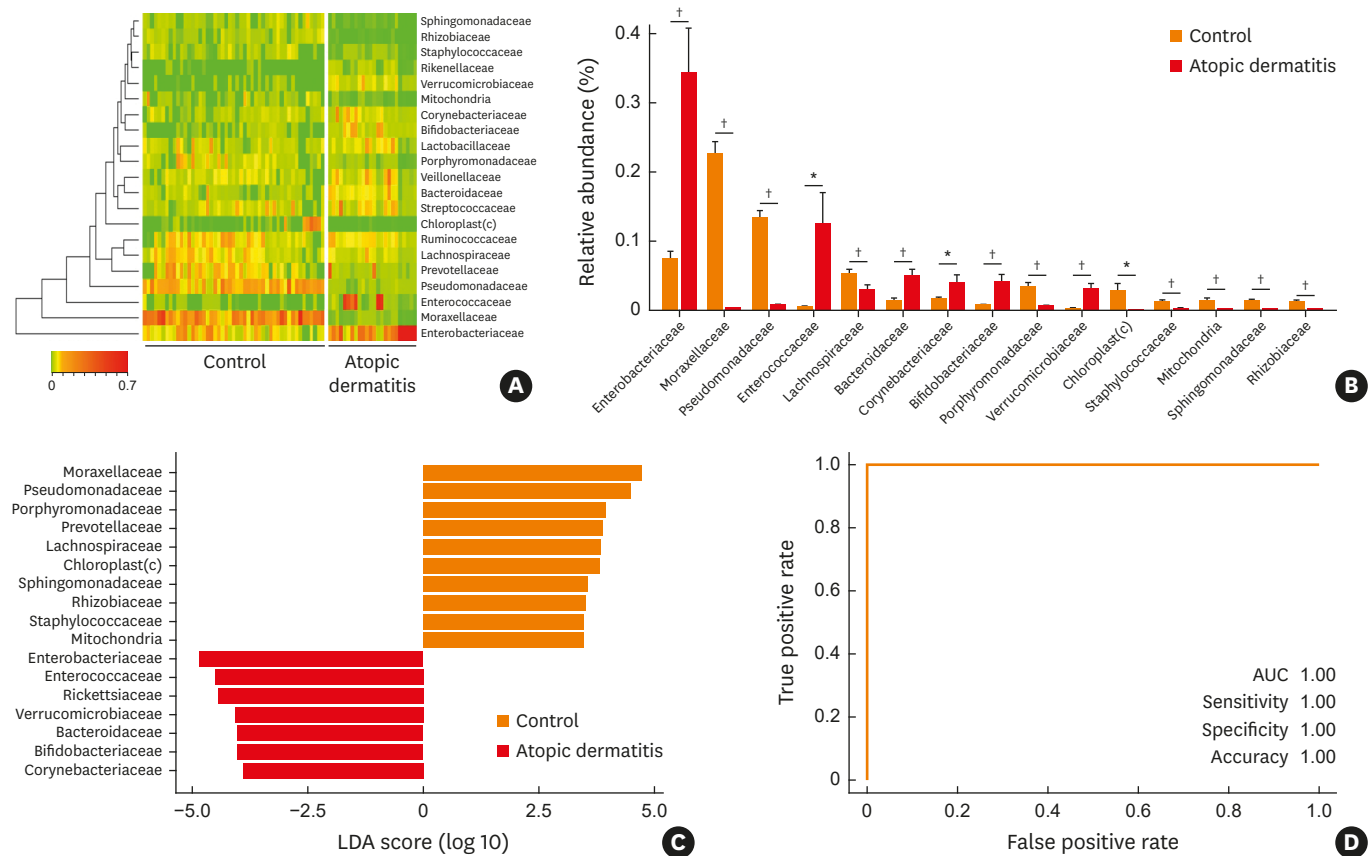
**Fig. 3.** Relative abundance of EVs in the sera of the control and AD groups at the order level. The most abundant serum microbial EV orders were expressed in (A) heatmap and significantly different orders plotted in (B) barplot. (C) LefSe-based order-level serum microbial EV biomarkers (LDA score > 3) were fitted in linear regression and expressed as (D) ROC curves.

EV, extracellular vesicle; AD, atopic dermatitis; LefSe, linear discriminant analysis effect size; LDA, linear discriminant analysis; ROC, receiver operating characteristic; AUC, area under the receiver operating characteristic curve.

\* $P < 0.05$  and † $P < 0.01$ .

of Moraxellaceae (22.6%–0.3%), Pseudomonadaceae (13.4%–0.7%), Lachnospiraceae (5.3%–3.0%), Porphyromonadaceae (3.4%–0.5%), Chloroplast(c) (2.8%–0.0%), Staphylococcaceae (1.2%–0.3%), Mitochondria (1.3%–0.1%), Sphingomonadaceae (1.3%–0.1%), and Rhizobiaceae (1.3%–0.0%) were shown to be decreased in AD patients; meanwhile, the portions of Enterobacteriaceae (7.6%–34.4%), Enterococcaceae (0.4%–12.6%), Bacteroidaceae (1.4%–5.0%), Corynebacterium (1.6%–4.1%), Bifidobacteriaceae (0.6%–4.2%), and Verrucomicrobiaceae (0.3%–3.2%) were shown to be increased (Fig. 4A and B). Through LefSe assessment, the key biomarkers determined to be increased in the healthy controls were Moraxellaceae, Pseudomonadaceae, Porphyromonadaceae, Prevotellaceae, Lachnospiraceae, Chloroplast(c), Sphingomonadaceae, Rhizobiaceae, Staphylococcaceae, and Mitochondria; whereas, Enterobacteriaceae, Enterococcaceae, Rikenellaceae, Verrucomicrobiaceae, Bacteroidaceae, Bifidobacteriaceae, and Corynebacteriaceae were shown to be decreased (Fig. 4C). Through model internal validation, family-level AD diagnostic model sensitivity, specificity, accuracy and AUC were all determined to be 1.00 (Fig. 4D).

Finally, 80 and 55 bacterial genera occupied more than 0.1% of the total abundance of healthy controls and AD patients, respectively. *Acinetobacter* and *Pseudomonas* were dominant in healthy controls, while *Escherichia-Shigella* and *Enterococcus* were dominant in AD patients. The 2 genera



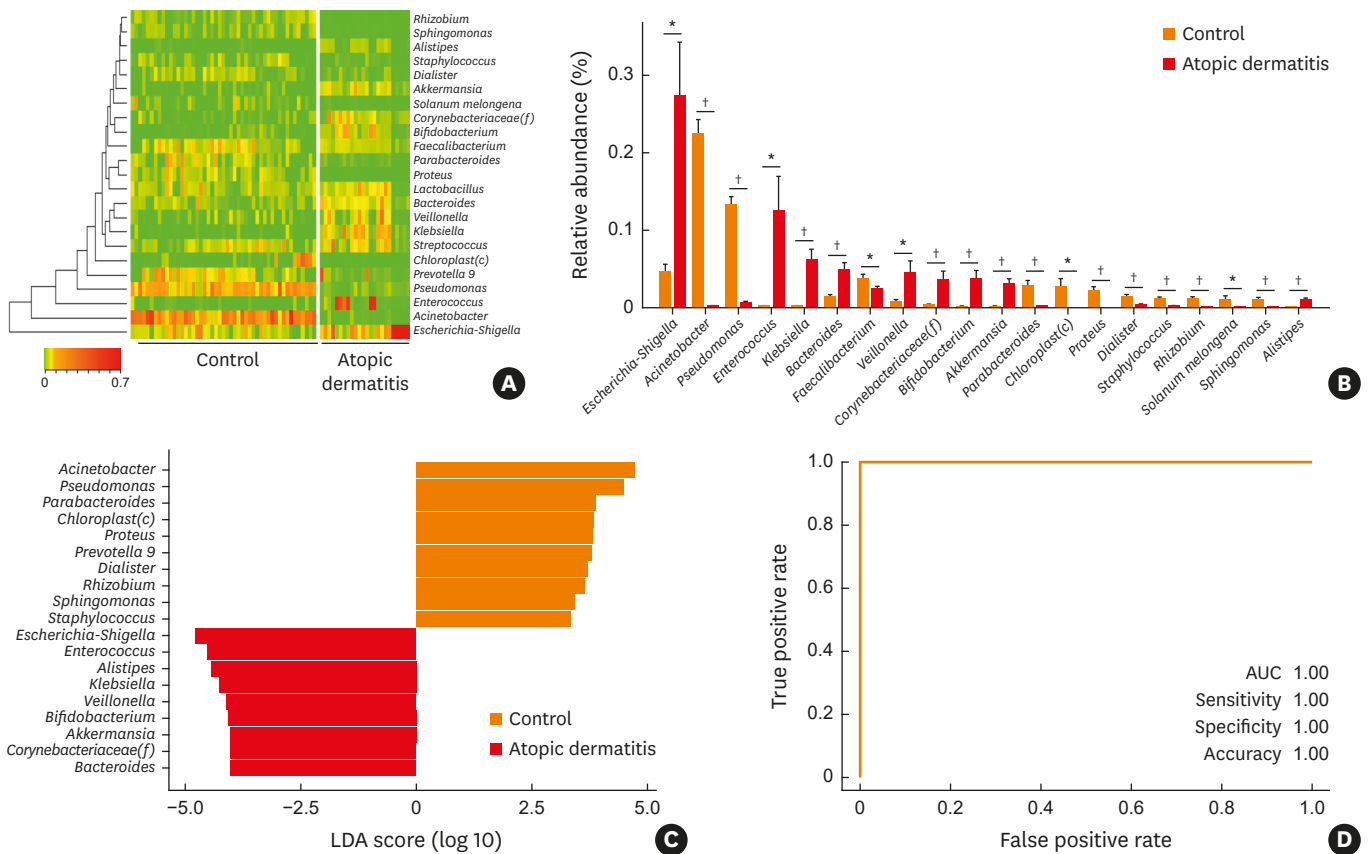
**Fig. 4.** Relative abundance of EVs in the sera of the control and AD groups at the family level. Abundant families of serum microbial EVs in AD and healthy control groups are shown in (A) heatmap with statistically significant differing families expressed in (B) barplot. (C) Family-level biomarkers were selected via LEfSe (LDA score > 3) and the resulting biomarkers used to create an AD diagnostic model expressed as (D) the ROC curve.

EV, extracellular vesicle; AD, atopic dermatitis; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis; ROC, receiver operating characteristic; AUC, area under the receiver operating characteristic curve.

\* $P < 0.05$  and † $P < 0.01$ .

dominant in each group accounted for over 35% of bacterial abundance. Genera that showed a significant decrease in abundance in the AD patients were *Chloroplast(c)*, *Proteus*, *Acinetobacter*, *Rhizobium*, *Pseudomonas*, *Sphingomonas*, *Parabacteroides*, *Solanum melongena*, *Staphylococcus*, and *Dialister* (more than 2-fold and  $P < 0.05$ ). Conversely, genera that were significantly increased in the AD patient samples included *Enterococcus*, *Klebsiella*, *Alistipes*, *Bifidobacterium*, *Akkermansia*, *Corynebacteriaceae(f)*, *Veillonella*, *Escherichia-Shigella*, and *Bacteroides* (more than 2-fold and  $P < 0.05$ ) (Fig. 5A and B). As a result of the microbiome analysis of serum samples, a total of 145 bacterial genera showed statistically significant ( $P < 0.05$ ) differential composition between the AD patients and the healthy control subjects, while 100 of these genera showed a greater than 2-fold change in relative abundance. Biologically and statistically significant genus-level biomarkers selected through LEfSe analysis included *Acinetobacter*, *Pseudomonas*, *Parabacteroides*, *Chloroplast(c)*, *Proteus*, *Prevotella*, *Dialister*, *Rhizobium*, *Sphingomonas*, and *Staphylococcus*, which were shown to be increased in the control group. Meanwhile, *Escherichia-Shigella*, *Enterococcus*, *Alistipes*, *Klebsiella*, *Veillonella*, *Bifidobacterium*, *Akkermansia*, *Corynebacteriaceae(f)*, and *Bacteroides* were decreased in the control group (over 1% in any group,  $P < 0.05$  and LDA score > 3). *Acinetobacter*, *Pseudomonas*, *Escherichia-Shigella*, *Enterococcus*, *Alistipes*, *Klebsiella*, *Veillonella*, *Bifidobacterium*, and *Akkermansia* were determined to be key biomarkers as they all had LDA scores greater than 4 (Fig. 5C). The final genus-level AD diagnostic model was calculated





**Fig. 5.** Relative abundance of EVs in the sera of the control and AD groups at the genus level. The most abundant serum bacterial genera were expressed in (A) heatmap and those genera that significantly differed between the control and AD were plotted in (B) barplot. (C) LefSe analysis was used to determine biologically and statistically significant genera-level biomarkers which were then used to create an AD diagnostic model expressed as (D) the ROC curve. EV, extracellular vesicle; AD, atopic dermatitis; LefSe, linear discriminant analysis effect size; LDA, linear discriminant analysis; ROC, receiver operating characteristic; AUC, area under the receiver operating characteristic curve. \* $P < 0.05$  and † $P < 0.01$ .

using multiple logistic regression of the biomarkers selected through LefSe analysis. The results of the model internal validation showed that sensitivity, specificity, accuracy and AUC were all expressed as 1.00 (Fig. 5D).

At the species level, *Enterococcus durans*, *Klebsiella* sp. A4-KS2, *Pseudomonas* sp. SGB188, *Bifidobacterium animalis*, and *Proteus* sp. NC were altered significantly between the control and AD patient groups, whereas the species of other genera were not profiled (Supplementary Fig. S3). In addition, AD patient samples were clustered into 3 groups. The ages of Class I, II, and III were  $11.6 \pm 6.3$ ,  $6.8 \pm 3.3$ , and  $6.0 \pm 4.5$ , respectively (Supplementary Fig. S4).

## DISCUSSION

Findings on the relationship between microbial components in blood and chronic diseases have previously been published not only by us but by other groups. Despite the technical difficulties of cultivating bacteria from blood, many previous studies have reported successful culture, and microscopic and NGS detection of numerous bacteria from the blood of healthy subjects.<sup>21-24</sup> However, analysis of the microbiome present in the blood, including microbial

EVs, has not been extensively reported.<sup>25</sup> Successful quantification and characterization of the taxonomic profile of blood have been made possible by our group through high-throughput sequencing technology and optimization of a specific pipeline of targeted metagenomics.<sup>25</sup> The metagenomic method of this study is reliable due to the fact that the number of reads is sufficient and that the proportion of unassigned taxa is 1.7% in healthy controls and 4.5% in the AD patients. Unassigned taxa are assumed to be EVs derived from human cells and cell-free DNA contained in the serum. Results of the previous blood metagenomic studies have shown that Proteobacteria account for over 80% of the total proportion, followed by Actinobacteria, Firmicutes, and Bacteroidetes.<sup>25,26</sup> In the present study, although the dominant blood microbiome phyla were likewise Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes, the Proteobacteria proportion was less than 80%. Possible reasons for this discrepancy may include the place of residence as well as the process of isolating EVs used in this study.

Until now, microbiome studies for AD have mainly targeted the skin microbiome. However, we here assessed AD patients' EV microbiome in serum for the first time. The skin microbiome interacts with other microbes and human cells as well as with the human immune system in multiple ways that mediate the risk of disease.<sup>27,28</sup> However, it is evident that microbial EVs in the blood play a more critical role in diseases such as AD due to their direct interaction and effect on the human immune system.<sup>29</sup> Furthermore, microbial EVs derived from the blood have a wider range of activity, since they circulate throughout the entire body.<sup>30</sup> Altered bacteria-derived EV composition may have either a causal relationship to disease outcomes or be altered as a result of that outcome. Nano-sized EVs can pass through the epithelial cell barrier in the gut, unlike larger intestinal bacteria. EVs that have passed through the epithelial barrier are then transported throughout the body through the bloodstream, affecting the host's organs and tissues.<sup>30</sup> EVs within the host's bloodstream exhibit variable correlation with tissue metagenomics in certain taxa of bacterial EVs showing positive, negative, or no correlation with tissue metagenomics. As previously stated, it is not certain whether these correlated EVs are the cause of what transpired in the tissues, or whether they are the result of altered tissue bacterial states that differentially release bacterial EVs into the bloodstream. Ultimately, according to our findings, it is believed that microbial EV composition contributes to tissue metagenomics. However, further studies are necessary to definitively conclude the specific cause and effect relationship between microbial EV composition and tissue metagenomics.

It is now evident that EVs released by bacterial and eukaryotic cells are an important mode of intercellular communication, influencing both neighboring and distant cells.<sup>31-33</sup> Previous studies targeting the skin fluid for metagenomic analysis showed that *Staphylococcus*, *Pseudomonas*, *Streptococcus*, *Acinetobacter*, and *Gemella* proportions in AD groups were higher in the AD patients than in the healthy controls, while *Alcaligenaceae(f)*, *Sediminibacterium*, *Lactococcus*, *Rhodococcus*, *Lactobacillus*, *Methylobacterium*, *Leuconostoc*, and *Haemophilus* proportions were significantly lower in the AD groups than in the healthy controls at the genus level.<sup>16,34,35</sup>

Another previous study that targeted the gut microbiome showed that *Bifidobacteria* had a lower proportion in the non-atopic patients than in the AD patients, while *Staphylococcus*, *Enterobacteriaceae(f)*, and *Clostridium* were higher.<sup>36-39</sup> Furthermore, in a previous urine microbiome study, *Pseudomonas*, *Streptophyta*, *Propionibacterium*, *Methylobacterium*, *Enterobacteriaceae(f)*, and *Corynebacterium* were higher in the AD patients compared to the control group, whereas *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Bradyrhizobium* were lower.<sup>40</sup> In the present study, *Streptococcus*, *Bifidobacterium*, and *Escherichia-Shigella* were increased, and

*Lactobacillus*, *Acinetobacter*, *Pseudomonas*, *Haemophilus*, and *Propionibacterium* were decreased in the AD patients compared to the healthy controls. Taken together, the present data suggest that the microbiome profiles of AD and healthy subjects vary according to the sampling area and sample type as seen in many previous studies. Biomarkers based on metagenomic analysis tend to differ between different sample types such as blood, urine, stool, and skin fluid. In this study, only serum samples were used for biomarker selection to minimize variation associated with different sample types. No previous studies have shown a strong correlation of the microbial EV microbiome composition between different sample types. In this study, only serum samples were used, and biomarkers were selected based on the metagenomic analysis of microbial EVs derived from serum. Future studies should be conducted on other sample types of AD patients and healthy people to contrast the diagnostic strength of microbial EV biomarkers associated with different sample types.

We have discovered biomarkers for diagnostic assessment using microbial EVs from AD patient sera, and these might be biomarkers for treatment and risk assessment through further studies. Several studies have suggested that the microbiome can be used as a source of biomarkers for the prediction and early diagnosis of diseases,<sup>41,42</sup> and a variety of microbiome-based methods have been reported for biomarkers selection.<sup>41,43</sup> In this study, the diagnostic models developed based on serum microbial EV analysis showed high accuracy, sensitivity, specificity, and overall model strength based on internal validation; however, due to the lack of samples, we did not perform external validation. Also, we could not include other parameters, such as BMI or family history as covariates in this study, due to the unavailability of the relevant clinical subject demographic information of the subjects utilized for diagnostic model development. We are continuously collecting more samples from both healthy subjects and AD patients with a focus on obtaining as much thorough clinical information and background as possible for the inclusion of more covariables in future microbiome-based disease diagnostic model development. Future studies should include such information as medication history, comorbidity, and intake of prebiotics, probiotics, or antibiotics to improve the diagnostic capability of the serum EV-based model. We suggest that diagnostic methods could be more accurate with serum EV microbiome and the addition of more detailed clinical information. Furthermore, we suggest the inclusion of more samples and more clinical information would facilitate the observation of higher clinical diversity between different microbiome composition clusters.

Here, we offer evidence that the microbiome might be a critical factor in disease diagnosis. Through microbiome utilization, objective diagnostic methods could be attained. EV-based metagenomic markers, which could be non-invasively used, might diagnose and detect the risk of AD development. While this study strongly supports the potency of serum EV microbiota-based diagnostics, further clinical research and external validation are mandatory to confirm the efficacy of our diagnostic models.

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## SUPPLEMENTARY MATERIALS

### Supplementary Fig. S1

Relative abundance of EVs in the sera of the control and AD groups at the phylum level. Differential bacterial composition was demonstrated in a (A) heatmap and (B) bar plot of phyla that had significantly different relative abundance. (C) LEfSe analysis was used to determine biologically and statistically significant biomarkers with LDA scores greater than 3 and the resulting phylum-level biomarkers were fit using linear regression analysis and expressed as a (D) the ROC curve.

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### Supplementary Fig. S2

Relative abundance of EVs in the sera of the control and AD groups at the class level. Differing serum microbial EV classes were expressed in (A) heatmap and (B) barplot. Class-level (C) biomarkers with LDA scores greater than 3 were determined via LEfSe analysis and the resulting diagnostic AD model using class-level biomarkers expressed as (D) the ROC curve.

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### Supplementary Fig. S3

Relative abundance of AD and control serum bacterial EVs at the species level (A). heatmap and significantly different orders plotted in a (B) bar plot.

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### Supplementary Fig. S4

Cluster of the AD patients according to serum EVs microbiome.

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