# Understanding the Gut Microbiota in Pediatric Patients with Alopecia Areata and their Siblings: A Pilot Study

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A cross-sectional study of 41 children aged 4–17 years with alopecia areata and 41 of their siblings without alopecia areata was conducted. A total of 51% had the Severity of Alopecia Tool scores in the range of 0–25%, 12% had scores between 26% and 49%, and 36% had scores between 75% and 100%. The fecal microbiome was characterized using shotgun metagenomic sequencing. A comparison of alpha and beta diversity yielded a small but statistically significant difference on the basis of Jaccard distance, which measures species presence and absence between samples. However, a follow-up analysis did not reveal the particular species that were present more often in one group. The relative abundance of one species, *Ruminococcus bicirculans*, was decreased in patients with alopecia areata relative to that in their sibling controls. An analysis of gene ortholog abundance identified 20 orthologs that were different between groups, including spore germination genes and genes for metal transportation. The associations reported in this study support a view of pediatric alopecia areata as a systemic disease that has effects on hair but also leads to internal changes, including differences in the gut microbiome.

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## INTRODUCTION

Alopecia areata (AA) is a T-cell–mediated autoimmune disease (AID) with unknown pathogenesis and no approved therapies (Gilhar et al., 2012; Rajabi et al., 2018). Genetic profiling of AA has shown deficiencies in the mechanisms of both peripheral and central tolerance (Coda and Sinha, 2011; Jabbari et al., 2016; Suárez-Fariñas et al., 2015). In other autoimmune disorders, such as rheumatoid arthritis and inflammatory bowel disease, many genetic and mechanistic parallels exist, and there is a growing body of data about the role of microbiota in onset and flares. AA risk genes are shared with many other AIDs such as rheumatoid arthritis, type 1 diabetes, celiac disease, systemic lupus erythematosus, multiple sclerosis, and psoriasis (Petukhova et al., 2010). The current off-label immune-suppressive therapies can put children at short-term risk for infection and long-term risk for malignancy. In similar autoimmune diseases, there has been increasing evidence that altering the bacteria of the gastrointestinal tract may mitigate the disease. Fecal microbiome transplantation has been successfully applied to control recurrent *Clostridioides difficile* infection and can be beneficial for inducing the remission of inflammatory bowel disease (Weingarden and Vaughn, 2017). Owing to its effectiveness in AIDs, there are ongoing randomized clinical trials of fecal microbiome transplantation in patients with rheumatoid arthritis (Zhang, 2019). In 2017, a case study reported hair growth in two young adults with alopecia universalis (and inflammatory bowel disease) treated with fecal transportation for secondary *C. difficile* infections. Both subjects previously were refractory to standard therapy (Rebello et al., 2017). The gut microbiota has been analyzed in adults with AA but not in pediatric populations. In this study, we address the knowledge gap by conducting a cross-sectional study evaluating the microbiome of 41 children aged 4–17 years with AA and their siblings aged 4–17 years without AA as control subjects.

## RESULTS

Of 41 children with AA, 11 were males, and 30 were females. Ages ranged from 4–17 years, with 22% of them aged 4–7 years, 27% of them aged 8–11 years, 34% of them aged 12–15 years, and 17% of them aged 16–17 years. Of them, 29 of 41 (71%) subjects identified as Caucasian. AA severity ranged from mild to severe: 51% had Severity of Alopecia Tool scores in the range of 0–25% (mild), 12% had scores between 26% and 49%, and 36% had scores between 75% and 100% (severe). The most common comorbidity among subjects with AA was atopy (26.8%), including eczema, seasonal allergies, food allergies, and asthma. The diet was predominantly Western/meat eaters (83%), but a small portion was vegan (2%), vegetarian (2%), gluten free (5%), or dairy free (7%) (Table 1).

We characterized the fecal microbiome of all subjects using shotgun metagenomics and recovered 2.7 million read

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Abbreviations: AA, alopecia areata; AID, autoimmune disease

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**Table 1. Patient Characteristics (N = 41)**

| Characteristics | Value |
|---|---|
| Sex assigned at birth, n (%) | |
| Male | 11 (26.8) |
| Female | 30 (73.2) |
| Age, y, n (%) | |
| 4–7 | 9 (22.0) |
| 8–11 | 11 (26.8) |
| 12–15 | 14 (34.1) |
| 16–17 | 7 (17.1) |
| Race, n (%) | |
| American Indian or Alaska Native | 0 (0) |
| Asian | 5 (12.2) |
| Black or African American | 2 (4.9) |
| Indian | 1 (2.4) |
| Native Hawaiian or Other Pacific Islander | 0 (0) |
| White/Caucasian | 29 (70.7) |
| Other | 3 (7.3) |
| Refused | 1 (2.4) |
| Diet, n (%) | |
| Western | 34 (82.9) |
| Vegan | 1 (2.4) |
| Gluten free | 2 (4.9) |
| Dairy free | 3 (7.3) |
| Vegetarian | 1 (2.4) |
| Comorbidities, n (%) | |
| Yes | 26 (63.4) |
| No | 15 (36.6) |
| Conditions reported, n (%) | |
| Belly pain | 6 (14.6) |
| Food allergy | 6 (14.6) |
| Constipation | 3 (7.3) |
| Eczema | 2 (4.9) |
| Asthma | 2 (4.9) |
| Down syndrome | 2 (4.9) |
| Seasonal allergy | 1 (2.4) |
| Diarrhea | 1 (2.4) |
| Lactose intolerant | 1 (2.4) |
| GERD | 1 (2.4) |
| Autism | 1 (2.4) |
| Headaches | 1 (2.4) |
| Sarcoidosis | 1 (2.4) |
| Cerebral palsy | 1 (2.4) |
| SALT score, n (%) | |
| 0–25 | 21 (51.2) |
| 26–49 | 5 (12.2) |
| 50–74 | 0 (0) |
| 75–100 | 15 (36.6) |

Abbreviations: GERD, gastroesophageal reflux disease; SALT, Severity of Alopecia Tool.

pairs per sample (median value) after removal of low-quality sequences and human DNA (Figure 1). Our statistical analyses of microbiome diversity and abundance accounted for sibling pairs and included age as an additional variable. Microbiome alpha diversity, assessed by species richness and Shannon diversity, was not different between subjects with AA and their sibling controls (Figure 2a). Likewise, an analysis of Bray–Curtis distance between fecal microbiomes did not reveal systematic differences in the abundance of bacterial species (Figure 2b). However, an analysis of Jaccard distance, which measures the fraction of species present in one sample but not in the other, indicated a small but statistically significant difference (permutational ANOVA test, $R^2 = 0.014$, $P = 0.01$) (Figure 2b). Although the effect size for AA was smaller than the estimated effect size for age, we nonetheless searched for species-level taxa that were differentially present or absent between the study group and matched control subjects. We identified 32 bacterial species with nominal *P*-values lower than 0.05, but none were statistically significant after correction for multiple comparisons (Figure 3).

We next examined the taxonomic and gene function signatures of the microbiome in children with AA. Our search for taxa with relative abundance differences between patients with AA and their sibling controls identified one species, *Ruminococcus bicirculans*, with lower relative abundance in patients with AA (linear mixed model, $P = 0.02$) (Figure 4a). Our tests of bacterial gene ortholog abundance yielded 20 genes that differed between patients with AA and their sibling controls ($P < 0.05$ for all) (Figure 4b). The relative abundance of two spore germination genes, *gerKA* and *gerKC*, were decreased in children with AA, whereas two genes for metal transportation, *fbpA* and *ctpC*, were increased. We found one multidrug resistance gene, coding for a transporter protein, which had a higher relative abundance in children with AA. Thus, our analysis of bacterial species and gene orthologs revealed minor but statistically significant microbiome differences in children with AA.

## DISCUSSION

Although we identified only minor differences in this study, our findings could have implications for the role of the microbiome in pediatric AA. We hypothesize that a much larger study would be needed to identify which bacterial species, if any, might account for the observation in Jaccard distance. In adults with AA, a predictive model based on the number of bacterial counts of *Parabacteroides distasonis* and *Clostridiales vadin BB60* group was used previously to predict disease status with 80% accuracy (Moreno-Arrones et al., 2020). The initial presentation of AA is typically the ages of 4–6 years in pediatric patients, which is just at the time that fecal microbiota transitions from very varied to more similar to adult microbiota (Avershina et al., 2016; Gilhar et al., 2012; Ficaro et al., 2020; Rajabi et al., 2018). In early childhood (under age 4 years), the gut microbiota is characterized by relatively low stability and high responsiveness toward influencing factors. Influencing factors include the diet, which has major implications for the establishment of the gut microbiota (Borde and Åstrand, 2018). This may explain why genetically susceptible children do not have first episodes of alopecia until ages 4–6 years and some later. Influencing factors may also explain the difference in taxa between subjects with alopecia and their sibling control group.

The shift of the microbiota from early childhood to adulthood may be a key for understanding the host–microbiota interaction in patients with AA.

In this study, we found small differences in species and gene function abundance in the gut microbiome of patients with AA (Figure 4), which may serve as a nucleus for an

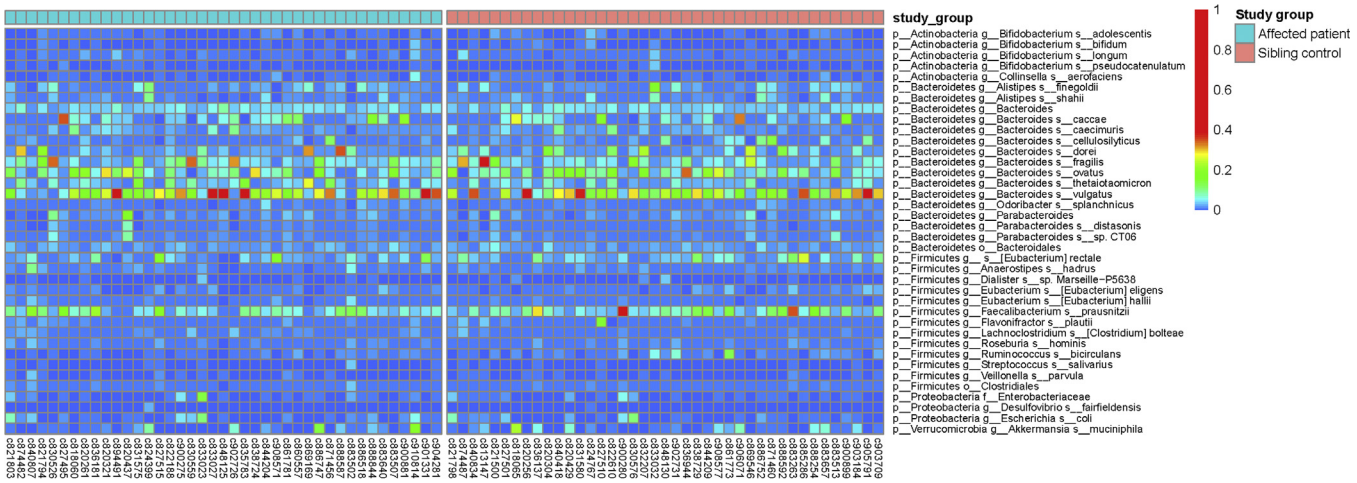



**Figure 1. Heatmap.** Heatmap of bacterial species relative abundance in children with AA and their sibling controls. The color scale is graded to show additional detail at low relative abundance. AA, alopecia areata.

improved understanding of environmental triggers such as diet and antibiotic exposure in pediatric AA. *R. bicirculans* has been reported as decreased in other autoimmune diseases (Bibbò et al., 2020; Forbes et al., 2016) and has the selective capacity to aid with uptake of nutrients from polysaccharides (Dassa et al., 2014). Consequently, we might hypothesize that this observation is diet associated. We know that dietary patterns play a role in genetically susceptible hosts, but this might provide evidence for why some are more likely to develop the disease. As for the differences in bacterial gene abundance, we identified genes that were associated with spore germination and multidrug resistance. Most members of the Clostridia family are spore-forming bacteria, including *R. bicirculans.* Thus, the result is broadly compatible with our taxonomic results. Our observation of increased abundance among multidrug-resistant genes might suggest a microbial response to antibiotic exposure. Future studies may explore this association by collecting long-term antibiotic exposure data in pediatric subjects with AA.

## MATERIALS AND METHODS

The study was approved by the Children's Hospital of Philadelphia (PA) Institutional Review Board (#18-01550), and parents/guardians gave their written informed consent to participate, and children provided assent. Patients with AA aged 4−17 years were identified through medical record review. Children were excluded if antibiotics were taken in the last 6 months. Eligible subjects were provided with home stool collection kits with instructions for putting a tray on the toilet and then transferring to a sterile container and package.

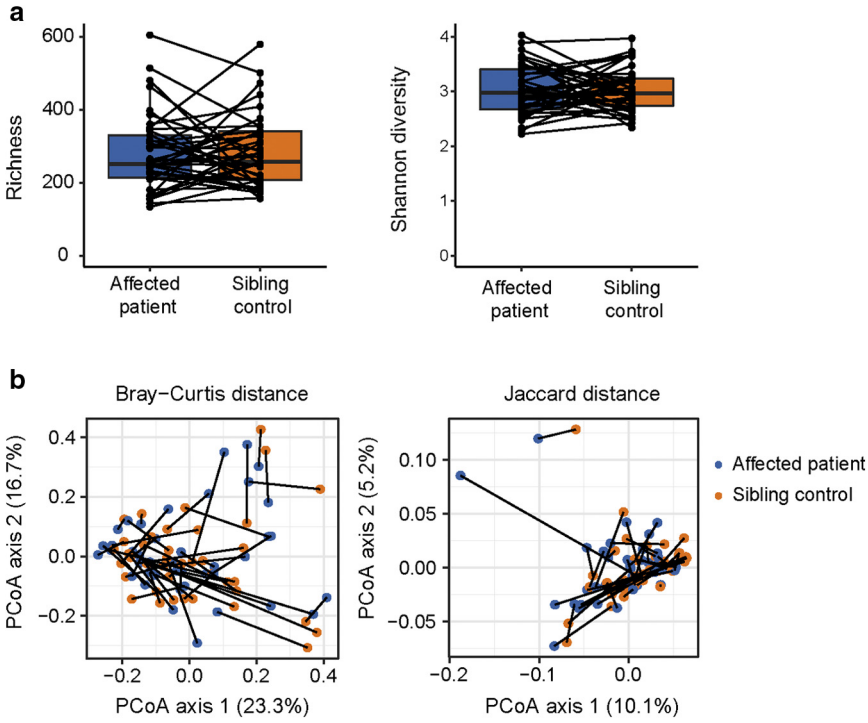



**Figure 2. Gut microbiome diversity and composition.** (**a**) Bacterial species richness and Shannon diversity. (**b**) Comparison of overall gut microbiome community between samples by Bray–Curtis (abundance-weighted) and Jaccard (unweighted) distance. Lines connect samples from sibling pairs. PCoA, principal coordinates analysis.

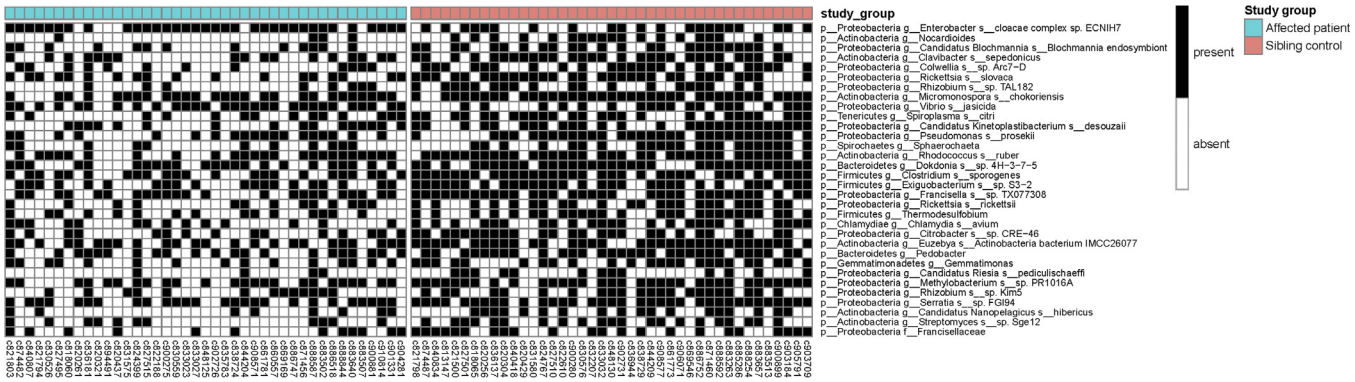
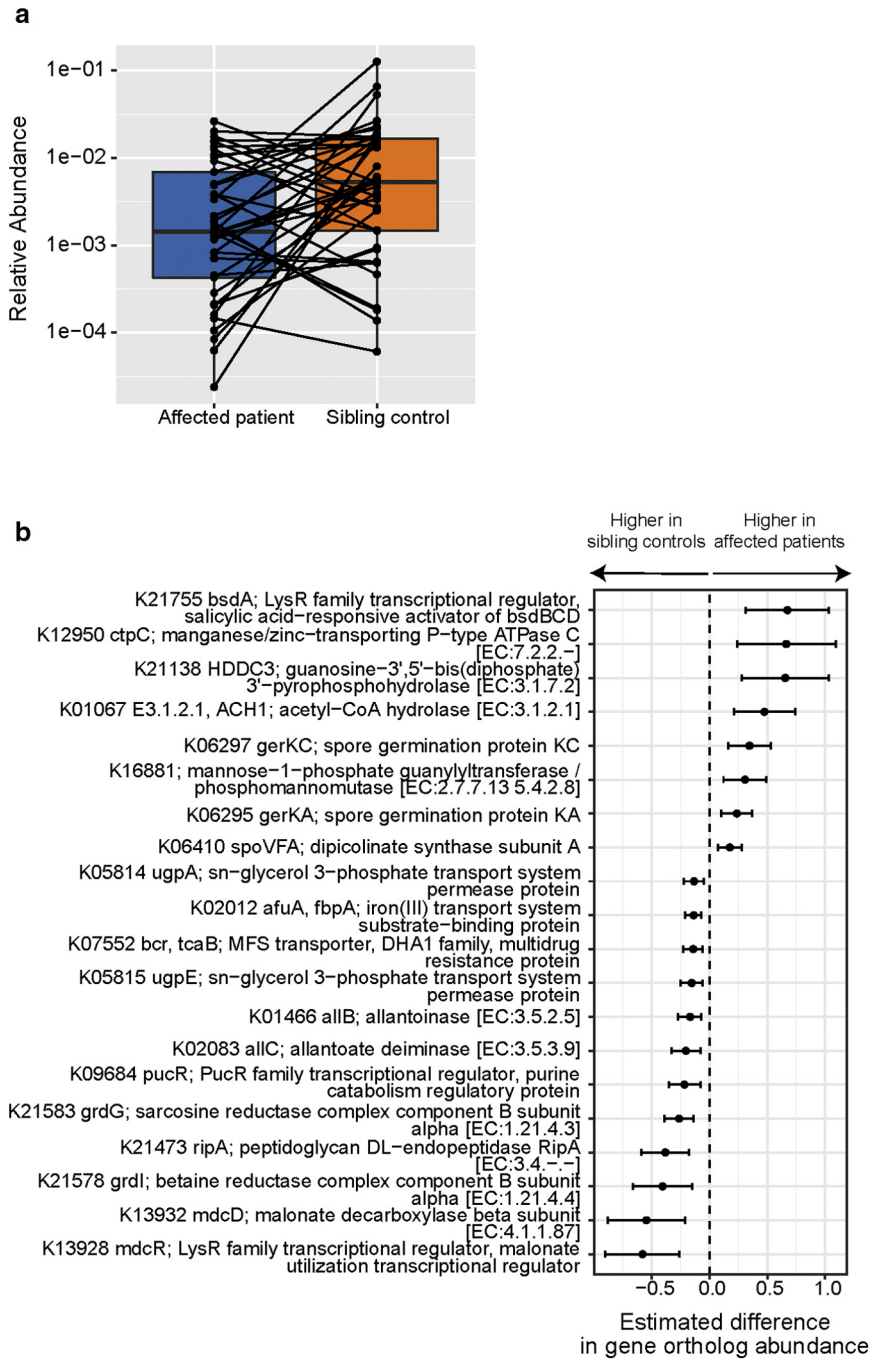



**Figure 3. Candidate species.** Candidate species were identified as present (black square) or absent (white square) more often in the gut microbiome of children with AA (uncorrected $P < 0.05$, corrected $P > 0.05$ for all species shown). AA, alopecia areata.

**Figure 4. Species and gene ortholog abundance in the gut microbiome.** (**a**) Relative abundance of *Ruminococcus bicirculans* in patients with AA and their sibling controls. Lines connect samples from sibling pairs. (**b**) The estimated effect size for relative abundance differences in 20 gene orthologs where statistically significant relative abundance differences were identified. Effect size estimates were determined from linear mixed models of log-scaled gene ortholog abundance versus age and disease status. Positive estimates correspond to genes that are higher in affected patients. Error bars extend to one standard error above and below the estimate. AA, alopecia areata.



Stool kits were mailed to Children's Hospital of Philadelphia and were then processed in the Children's Hospital of Philadelphia Microbiome Center.

Shotgun metagenomic sequencing was carried out in the Children's Hospital of Philadelphia Microbiome Center. The DNeasy PowerSoil Kit (Qiagen, Germantown, MD) was used for DNA extraction. The NexteraXT DNA Library Preparation Kit (Illumina, San Diego, CA) was used to generate DNA libraries for shotgun metagenomic sequencing. DNA sequencing was carried out on a HiSeq 2500 instrument, producing 125 base pair paired-end sequence reads. Additional samples of DNA-free water and DNA extraction blanks were processed in parallel with the experimental samples to assess reagent and laboratory contamination.

Bioinformatics analysis was conducted with the Sunbeam metagenomics pipeline (Clark et al., 2019). Taxonomic assignments were generated with Kraken, version 2.1.1. (Wood et al., 2019). The Kyoto Encyclopedia of Genes and Genomes database was used to assign and classify gene orthologs (Kanehisa and Goto, 2000). Species richness or the number of bacterial species per sample was estimated using a sequencing depth of 10,000 reads per sample. Shannon diversity or the abundance-weighted species diversity was calculated using a natural logarithm. Community-level differences between sample groups were assessed using Bray–Curtis distance and Jaccard distance between samples. Sample−sample distances were tested with permutational ANOVA, where permutations were restricted to randomize samples within sibling pairs (Anderson, 2001). When multiple tests were conducted, *P*-values were corrected using the method of Benjamini and Hochberg (1995) to control for a false discovery rate of 5%.

### Data availability statement

The data that support the findings of this study are available on request from the corresponding author, LCS. The data are not publicly available because they contain information that could compromise the privacy of research participants.

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### AUTHOR CONTRIBUTIONS

Conceptualization: LCS; Data Curation: LCS, SR, KB, JJL; Formal Analysis: KB, JJL, WH; Funding Acquisition: LCS; Investigation: LCS, SR, KB, JJL, WH; Methodology: KB, JJL, WH; Writing - Original Draft Preparation: LCS, SR; Writing - Review and Editing: LCS, SR, KB

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