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SUPPORTING INFORMATION

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Variations in oral microbiota and salivary proteomics reveal distinct patterns in polysensitized individuals

To the Editor,

Dynamics in microbiota-immune interactions have been associated with several atopic phenotypes at diagnosis and treatment.^{1,2} Additionally, dysbiosis of oral microbiota (OM) has been implicated in the pathogenesis of allergic diseases.³ Despite recent evidence of the associations between OM and oral mucosa immunity in peanut allergy,⁴ the potential links between OM and the host oral milieu in different sensitization patterns are overlooked. Here, we report a novel and multi-omic study using saliva as a useful tool to explore a distinct OM-oral environment relationship in polysensitized atopic individuals.

Unstimulated saliva was collected from healthy controls (HC; $n = 11$) and atopic adults with different sensitization patterns (AP; $n = 19$; cohort information shown in Table S1; for methods, see Appendix S1). Biochemical analysis first showed that the saliva from AP had significantly higher levels of total protein ($p < .0001$; Figure 1A-a) and protease activity ($p = .039$; Figure 1A-c) than the HC, while no significant differences existed in the two groups regarding salivary total IgE, IgG₁, and IgG₂ (Figure 1B). Multiplex cytokine analysis showed that the cytokines that could differentiate the 2 groups were MIP-1 α ($q = 0.06$) and FGF-basic ($q = 0.08$), two mediators shown to be involved in the development of

airway inflammation and allergic respiratory diseases (Figure 1C and Figure S1). Using unsupervised clustering, the cytokine profiles in AP could be separated into two distinct clusters (Figure 1D and Figure S2), with AP_{Clus2} showing relatively higher levels of IL-12, IL-4, IL-9, FGF-basic, and MIP-1 α , but lower levels of MCP-1 and IL-1RA than AP_{Clus1} (Table S2). Moreover, compared to HC, no cytokines were differentially expressed in the saliva of AP_{Clus1}, while in AP_{Clus2} vs. HC, the levels of up to 10 cytokines were statistically different. Of particular note, all the subjects in AP_{Clus2} were characterized by polysensitized conditions against several foods and aeroallergens (Figure 1D). A distinct salivary proteomic pattern seems to be linked to the phenotype of multi-sensitization in atopic individuals.

We next questioned that whether OM characteristics had a unique relationship to the multi-sensitized features in AP_{Clus2}. As shown in Figure 2A, the 4 groups of subjects (HC, AP as a whole, AP_{Clus1}, and AP_{Clus2}) harbored a similar oral bacterial burden. However, AP_{Clus2}, but not AP or AP_{Clus1}, displayed a significantly higher Shannon index than the HC ($p = .043$; Figure 2B). Further, bacterial relative abundance and β -diversity analysis clearly showed that the OM compositions in AP_{Clus2} were more dissimilar from HC than the OM of AP_{Clus1} (Figure 2C-E and Figure S3). This suggested a higher degree of bacterial imbalance in the oral cavity

of polysensitized individuals. LEfSe analysis further demonstrated possible loss and/or enrichment of certain community members in the OM of AP_{Clus2} (Figure 2F). It is worth noting that, compared to AP_{Clus1}, a series of taxa from the genera *Prevotella* were depleted in the OM of AP_{Clus2} (Figure 2F-b). The *Prevotella* spp. have been associated with the protection against atopic diseases including food allergy in human microbiome analysis.^{4,5} Finally, through correlation analysis, the abundance of the genera *Neisseria*, *Prevotella*, *Veillonella*, and *Streptococcus* was significantly correlated with the local secretion of Th1 (IL-1 β and TNF- α), IL-9, and eosinophil-related cytokines (Figure 2G). In peanut-allergic patients, it was also found that the depletion of oral *Prevotella* spp. and *Veillonella* spp. correlated with higher local secretion of Th2-cytokines, suggesting a role of relevant species in the modulation of T-cell tolerance.⁴ Further exploration of the role of these depleted or enriched oral bacteria in the development of multi-allergy is required. Overall, these data imply that variations in the microbiome-oral mucosa interactions play a crucial role in the polysensitized status of AP_{Clus2}.

Polysensitization is a distinct phenotype of atopic diseases, which disposes a challenge for the clinical interpretation and treatment of allergic patients.⁶ We next sought to bring a broader picture of the

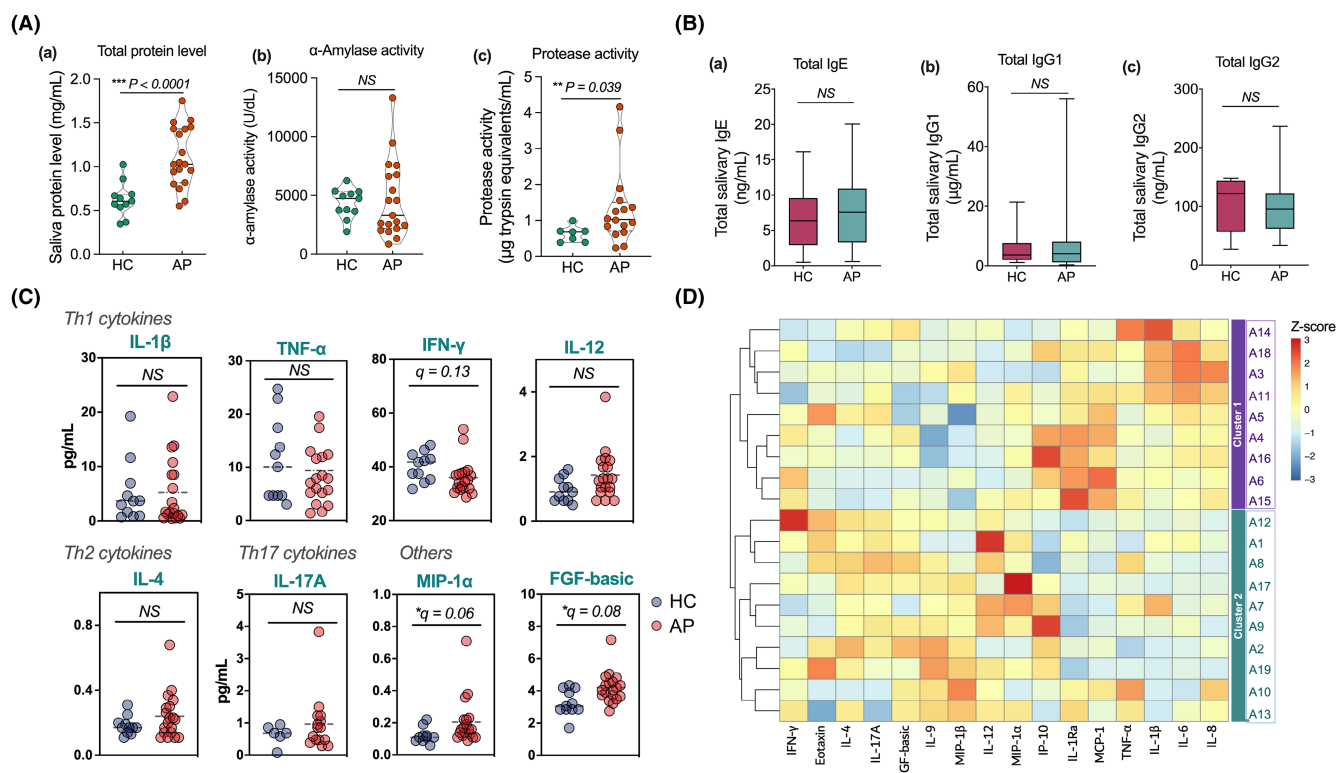


FIGURE 1 Distinct salivary cytokine patterns in polysensitized individuals. (A) Biochemical analysis of saliva samples: total protein level, α -amylase, and protease activities. (B) Salivary immunoglobulin levels: total IgE, IgG₁, and IgG₂. (C) Salivary multiplex cytokine analysis: Th1/Th2/Th17 cytokines and other cytokines that differentiated the two groups. (D) Unsupervised clustering of cytokine profiles in AP (A1-A19); subjects in purple, mono-sensitized to food or aeroallergens alone; subjects in green, polysensitized to foods and aeroallergens. HC, healthy controls ($n = 11$); AP, atopic patients ($n = 19$); NS, not significant. p values were determined using t-test or Mann-Whitney test, and corrected for multiple comparisons testing using Benjamini-Hochberg method, thus represented as q values. $*q < 0.1$, $**p$ or $q < 0.05$, $***p$ or $q < 0.001$

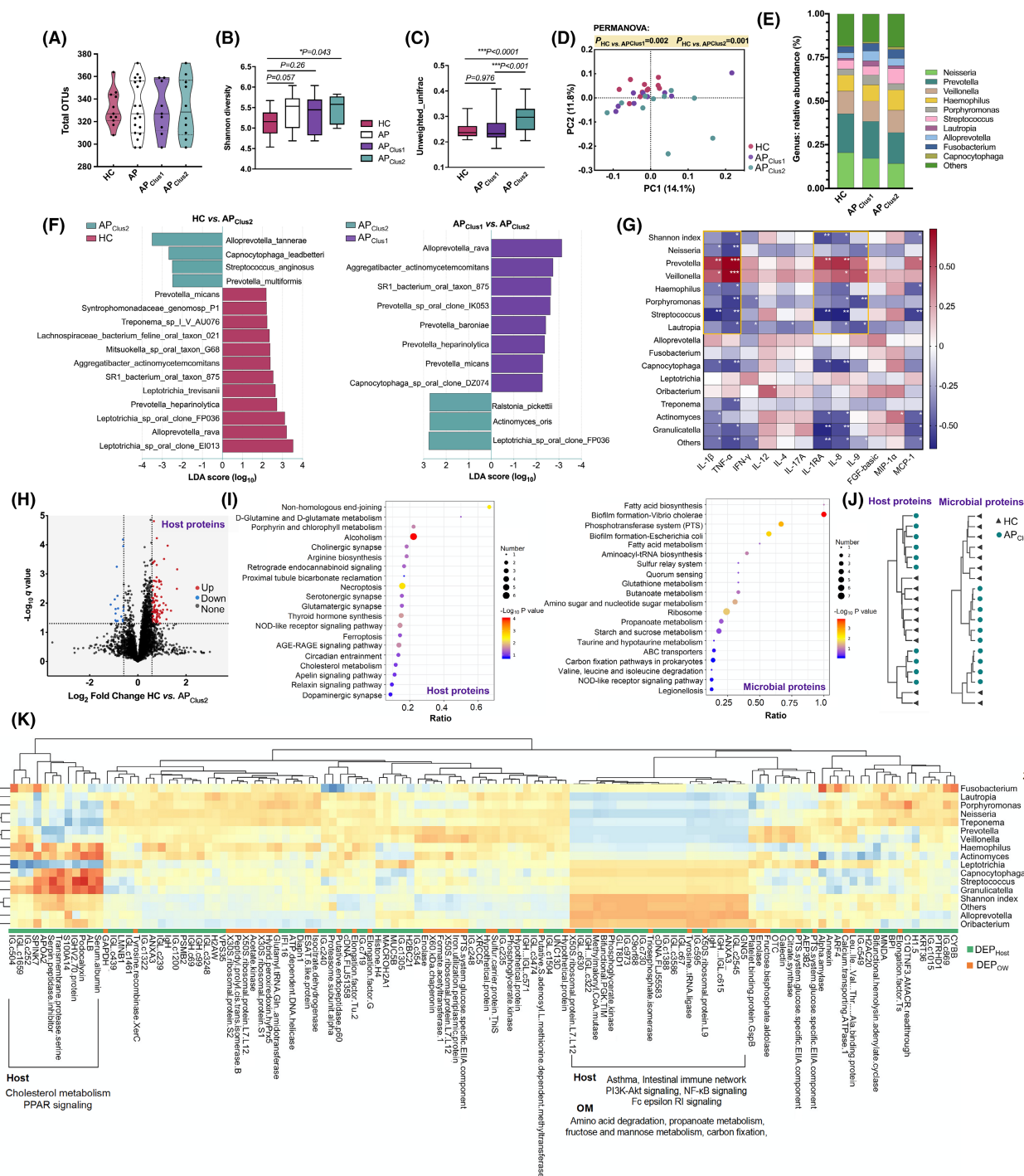


FIGURE 2 OM in AP_{Clus2} is more dissimilar from HC than the OM of AP_{Clus1} in association with disturbances in the oral micro-environment and saliva proteome. (A) Bacterial burden, as assessed by total OTU numbers. (B) Shannon index. (C) β -diversity, based on the unweighted Unifrac distance. (D) PCoA plot (unweighted_UniFrac distance), with each dot representing a sample colored by group. (E) Bacterial relative abundance at the genus level. (F) LEfSe analysis showing the species that best explain the differences between the OM of HC vs. AP_{Clus2} and AP_{Clus1} vs. AP_{Clus2}. (G) Heatmap showing the correlations between OM compositions and local secretion of immune cytokines in HC and AP. The 15 most abundant genera were shown and the cytokines include Th1, Th2, Th17 cytokines, and the cytokines that could differentiate AP from HC, and AP_{Clus2} from HC or AP_{Clus1}. (H) Volcano plots showing differentially expressed host-derived proteins (DEP_{Host}) in the saliva of HC vs. AP_{Clus2}. (I) KEGG pathway enrichment of DEP_{Host} and DEP_{OM}. (J) Hierarchical clustering based on the saliva proteomic pattern of host and microbial proteins in HC and AP_{Clus2}. (K) Clustered heatmap of Spearman correlation analysis between DEPs and OM compositions in HC and AP_{Clus2}. Only DEPs that correlate with at least one OM genera are shown. The pathways annotated were enriched by KEGG. OM, oral microbiota; DEPs, differentially expressed saliva proteins; DEP_{Host}, DEPs from the host; DEP_{OM}, DEPs from the OM; HC, healthy controls ($n = 11$); AP, atopic patients ($n = 19$); AP_{Clus1}, cluster 1 from the saliva cytokine clustering, subjects characterized by mono-sensitization ($n = 9$); AP_{Clus2}, cluster 2 from the saliva cytokine clustering, subjects characterized by polysensitization ($n = 10$). p values were determined using t -test or Mann-Whitney test, and corrected for multiple comparisons testing using Benjamini-Hochberg method, thus represented as q values. * p or $q < 0.05$, ** p or $q < 0.01$, *** p or $q < 0.001$.

disturbances in the oral environment of multi-sensitized subjects. Using a tandem mass tag-based quantitative proteomic approach, total 113 host proteins (DEP_{Host}) and 55 microbial proteins (DEP_{OM}) were found to be differentially expressed in the saliva of AP_{Clus2} vs. HC (Figure 2H-I and Figure S4). Clustering of both host and OM proteomic data displayed a comparable dendrogram in accordance with the disease state, again evidencing strong host-microbiome interactions (Figure 2J). Various correlations were also witnessed between the levels of DEP_{Host/OM} and the abundance of certain genera in OM (Figure 2K). Through clustering and KEGG analysis, part of the OM-associated DEP_{Host} was involved in asthma, intestinal immunity, and several key signaling pathways (e.g., PI3K-Akt and NF-κB signaling), and the OM-relevant DEP_{OM} were primarily related to carbohydrate and propanoate metabolism. The oral levels of short-chain fatty acids, including propanoate, were shown to be associated with altered immune profiles in food allergy.⁴ Moreover, the PPAR signaling was strongly correlated with some DEP_{Host}. It was reported that Th2 cells expressed high levels of PPAR-γ in response to aeroallergens, which suggested the contribution of PPARs to Th2-skewed pathologies (Appendix S1 Ref (9)).

In conclusion, this study performed a multi-dimensional analysis of the oral environment of atopic individuals, showing that polysensitization was a distinct clinical pattern that was closely related to the dysbiosis of OM, in association with certain disturbances in the local secretory and immunological balances. These data highlighted the role of complex OM-host oral milieu in the modulation of multi-morbidity and polysensitization, results of which would, on the one hand, benefit mechanistic studies on the functional assessment of OM in multi-allergic phenotypes, and on the other hand, highlight the oral cavity as an attractive site for preventive and therapeutic treatments for allergic multimorbidity based on microbiome manipulation.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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