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Selenomonas: a marker of asthma severity with the potential therapeutic effect

To the Editor,

The microbiome has come into the spotlight as one of the most important players regulating inflammation.¹ Dysbiosis in the asthmatic airway may partly explain asthma severity with regards to symptom frequency, lung function, and other clinical features.²⁻⁴ However, these findings were not consistent among studies. The purpose of this study is to understand the characteristics of the asthmatic airway by analysing the airway bacteriome, to identify the microbial genera associated

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with asthma clinical indices, and to investigate the polymicrobial interactions based on the asthma severity grouping.

A total of 155 subjects (31 non-asthma subjects, 124 asthmatics (47 non-severe and 77 severe)) were enrolled in this study (**Table S1, Figure S1A**). Data analysed by the ANCOM method showed that the asthma group had a significant reduction in the abundance of *Selenomonas*, *Campylobacter*, *JN713389g*, and *Saccharimonas* compared to non-asthma controls (**Table S2**).⁵ We analysed the bacteriome of asthmatics to identify specific bacterial genera associated with asthma-related clinical variables by performing zero-inflated negative binomial regression analysis (**Table 1**).⁶ Among these bacterial genera, the abundance of *Selenomonas* showed a significant correlation with Forced expiratory volume in 1 second (FEV1) and a negative association with uncontrolled asthma (higher Asthma control test (ACT) score, lower Asthma control questionnaire (ACQ) score).

To further identify the differences in the bacteriome community based on disease severity, the alpha and beta diversities were compared among the three groups. The Shannon index (**Figure S1B**) did not present significant differences among the groups. The beta diversity measures by normalized weighted UniFrac distance also showed that the bacteriome community was not significantly different among the groups (**Figure S1C**). At the genus level, *Streptococcus*, *Prevotella*, *Veillonella*, *Neisseria*, and *Haemophilus* accounted for 70% of the entire bacteriome population and their microbial composition was similar among the three groups (**Figure S1D**). The comparison of the relative abundance of each genus by ANOVA demonstrated decreased proportions of *Selenomonas*, *Campylobacter*, *Saccharimonas*, *Actinomyces*, *Peptostreptococcus*, and *Atopobium* in severe asthmatics compared to non-asthma controls and non-severe asthmatics (**Figure S2**). The OMiAT method showed that the relative proportions of *Selenomonas* and *Campylobacter* decreased as the disease severity increased (FDR adjusted $P = 0.011, 0.023$).

The relative abundance of *Selenomonas* showed a significant positive association with alpha diversity indices (Chao1 index and Shannon index) in asthmatics but not in non-asthma controls (**Figure S3**). The bacteria–bacteria interactions were calculated using a pairwise Spearman correlation (**Figure S4**). The co-occurrence network showed more microbial associations with higher complexity centred on *Selenomonas* observed in asthma groups, especially in more severe group. The number of pairs with a significant negative correlation coefficient was also increased in more severe asthmatics.

To investigate the biological significance of *Selenomonas* in asthma, we adopted a murine asthma model. Treatment during the sensitization period with *Selenomonas sputigena*, the most dominant Operational taxonomic unit (OTU) (48%) belonging to *Selenomonas* in human sputum, resulted in a significant reduction in airway hyperresponsiveness and a decrease in inflammatory cells present in bronchoalveolar lavage fluid (**Figure 1**). In addition, we saw a significant reduction in IL-5 and IL-13 secreting T_H2 populations in the lungs, suggesting that the *Selenomonas* may be able to mitigate the severity of asthmatic phenotypes.

We investigated the airway bacteriome of non-asthma controls and asthmatic patients with different disease severities. The *Selenomonas* was associated with different clinical features of asthma and its abundance was significantly reduced in asthmatics especially with severe disease. Such findings suggest that the lack of *Selenomonas* in the airway bacteriome can potentially be considered as a marker of severe asthma. Further studies validating the causal relationships between *Selenomonas* and the pathophysiological features of asthma may provide a platform for the future of a microbiota-based, personalized therapeutic approach to the treatment of asthma.

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CONFLICTS OF INTEREST

The authors declare that there is no potential conflict of interest.

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Table 1. Clinical factors associated with the abundance of genera in asthma patients (FDR < 0.1)

Phylum	Genus	Clinical factor	Estimate (log count)	P-value (FDR)
Firmicutes	<i>Selenomonas</i>	ACT score	0.175	0.015
		ACQ score	-0.606	0.024
		Day symptom	-1.161	<0.001
		FEV1_volume	0.001	0.098
	<i>Gemella</i>	Day symptom	0.200	<0.001
Fusobacteria	<i>Leptotrichia</i>	Day symptom	-0.221	0.077
Bacteroidetes	<i>Alloprevotella</i>	Day symptom	0.086	<0.001

ACT, asthma control test; ACQ, asthma control questionnaire; FDR, false discovery rate; FEV₁, forced expiratory volume in 1 s

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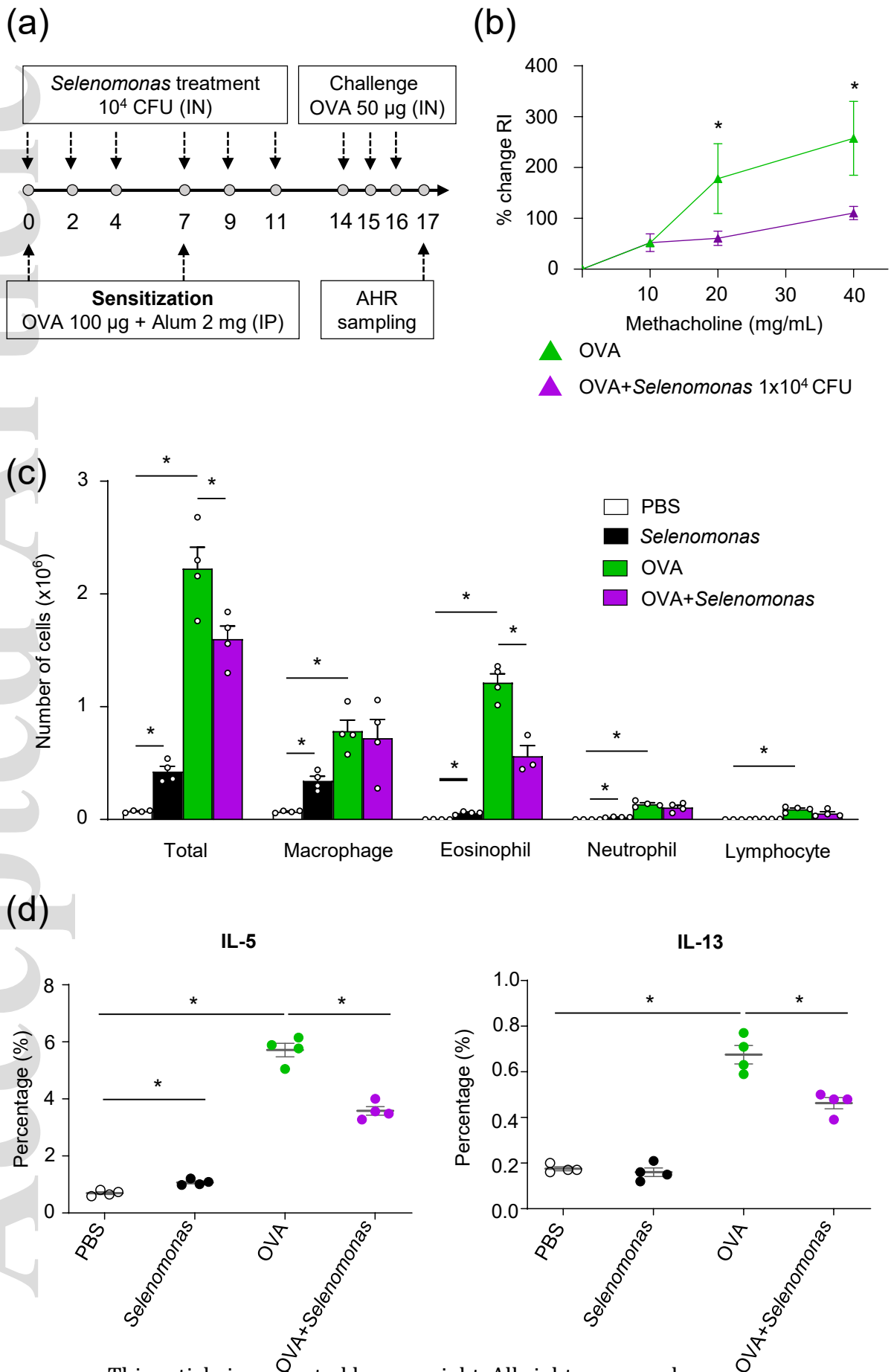
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Figure 1. *In vivo* treatment of *Selenomonas* reduces type 2 airway inflammation in mouse asthma model.

(a) A schematic illustration of ovalbumin (OVA) induced murine asthma model and protocol for pre-treatment of *Selenomonas*. **(b)** Responsiveness Index measurement after methacholine challenge of the lower airways (n=4 for each group). Sample groups were compared by unpaired t-test with multiple comparisons using the Holm-Sidak method. **(c)** Bronchoalveolar lavage fluid analysis of OVA induced asthma model. **(d)** Proportion of IL-5 and IL-13 secreting cell populations among CD4⁺ T cells was analysed using flow cytometry. Results are obtained from 4 independent experiments. * $P < 0.05$ significantly different between the indicated groups.

- Accepted Article
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Online methods

Study population

This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB approval number: H-1607-148-778), and informed consent was obtained from all patients. In total, 155 subjects (31 healthy subjects, 47 non-severe asthma, and 77 severe asthma patients) were enrolled.

All patients were nonsmokers or ex-smokers (less than 20 pack-years and quit smoking for at least 6 months). Those who were treated with antibiotics or who had experienced acute exacerbation event within the last three months were excluded from the study.

Asthma was defined as follows: (1) presence of chronic respiratory symptoms such as coughing, sputum, dyspnea, and wheezing, and (2) confirmation of airway reversibility or airway hyper-responsiveness (AHR). Airway reversibility was defined as an increase in forced expiratory volume in 1 second (FEV₁) of 12% by 200 mL or greater, when measured 15 minutes after inhalation of a short-acting β_2 agonist. AHR was defined as positive if less than 16 mg/mL of methacholine induced a 20% fall in FEV₁ in the bronchial provocation test.¹

Severe asthma was defined as an ACQ score of greater than 1.50 or an FEV₁ of 40-80% of the predicted value, despite use of a daily inhaled corticosteroid dose equivalent to 1,000 μ g or greater of fluticasone.²

Preparation of sputum samples for microbiome analysis

The enrolled subjects underwent sputum induction. After measurement of basal FEV₁, all participants were treated with 200 μ g of salbutamol (Ventolin[®]; GlaxoSmithKline, Brentford, England). To induce sputum production, 3% hypertonic saline solution was inhaled repeatedly, up to four times, at 5 min intervals using an ultrasonic nebulizer with an output

set at 4.5 mL/min (Omron Co., Tokyo, Japan). After each nebulization, the subjects rinsed their mouths and spat the sputum carefully into a Petri dish. Samples that contained squamous epithelial cells which exceeded 20% of the total cell count were excluded from the analysis.

DNA extraction, PCR amplification, and 16S rRNA gene sequencing

The whole sputum sample was an even mixture of fluid and cellular phases. Samples that contained squamous epithelial cells that exceeded 20% of the total cell count were excluded from the analysis.

Metagenomic DNA was extracted from 1 mL of this whole sputum sample using the FastDNA[®] SPIN Kit for DNA extraction kits (MP Biomedicals, USA). PCR amplification of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was subsequently performed using 3 µL of extracted DNA template and 25 µL 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Italy). The primers—318F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC-3') were used for 16S rRNA gene amplification. The PCR cycling conditions for V3-V4 hypervariable region of the bacterial 16S rRNA gene were as follows: 3 min at 95°C; 25 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, 5 min at 72°C, and holding at 4°C. The amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA), and the purified product was subjected to index PCR using the Nextera Index Kit (Illumina, USA) following the manufacturer's instructions. The resultant product was further

purified using AMPure XP (Beckman Coulter, USA). The prepared bacterial amplicon library was then quantified and sequenced using the MiSeq v3 platform (Illumina).

Sequencing data processing

To remove sequences with low quality scores, the raw sequence reads were pre-filtered using PRINSEQ.³ Adapter sequences were removed using CUTADAPT, and ambiguous bases were further trimmed using the FASTX-toolkit. Pair-end reads were merged using PEAR⁴ and then further filtered with PRINSEQ. Chimeric sequences and singletons were screened and reduced using USEARCH.⁵ A random sampling of 10,000 reads was performed to equalize the sequencing depth throughout the samples. The downstream data analysis was carried out using QIIME⁶ with the EzBioCloud 16S rRNA gene sequence database.⁷

***In vivo* treatment of *Selenomonas* on mouse asthma model**

The asthma model was established by injecting a mixture of 100 µg of ovalbumin (OVA) and 2 mg of alum intraperitoneally on day 0 and 7 followed by intranasal challenges with 50 µg of OVA on day 14, 15, and 16. The control group and the asthma group were treated either with PBS or 10⁴ CFU of heat-killed *Selenomonas* intranasally on day 0, 2, 4, 7, 9, and 11. Twenty-four hours after the final intranasal instillation, AHR was measured in response to increasing doses of methacholine (0, 5, 10, 20, and 40 mg/mL) by using the forced oscillation technique (FlexiVent System; SCIREQ, Montreal, Quebec, Canada). After measurement of AHR, bronchoalveolar lavage (BAL) fluid and lung tissue were collected from sacrificed mice. BAL cell slides were stained with Diff-Quik (Sysmex Co., Kobe, Japan), and at least 300 cells per slide were evaluated to obtain a differential leukocyte count. Mouse lungs were diced into small pieces by using razor blades. Minced lung tissues were incubated in 5 mL of RPMI1640 with 10% Type IV collagenase (Worthington Biochemical Corporation, Lakewood, NJ) at 37°C for 90 minutes and sorted through sterile cell strainer for single cell

preparation. Cells were blocked with Fc receptor binding inhibitor antibody at room temperature for 5 minutes, and then incubated with fluorochrome-labeled antibodies directed against cell surface markers for 30 minutes at 4°C. The antibodies used are as follows; FITC-conjugated anti-CD3 (Biolegend), BV785-conjugated anti-CD4 (Biolegend), PE-conjugated anti-IL-13 (eBioscience, San Diego, CA) and APC-conjugated anti-IL-5 (Biolegend). Flow cytometry was performed with the BD LSRFortessa X-20 and analyzed by using FlowJo (version 10.6) software.

Table S1. Baseline characteristics of enrolled subjects

	Non-asthma (n = 31)	Non-severe asthma (n = 47)	Severe asthma (n = 77)	P-value*
Female (%)	54.8	70.2	57.1	0.272
Age (years)	51.7 ± 8.0	58.9 ± 8.8	56.3 ± 9.3	0.003
BMI (kg/m ²)	23.9 ± 3.4	24.0 ± 2.9	24.7 ± 4.1	0.466
Ex-smoker (%)	0	4.3	1.3	0.032
Antibiotics in 6 months (%)	0	14.9	29.9	< 0.001
Oral steroids in 6 months (%)	3.2	27.7	58.4	< 0.001
Frequency of SABA use	NA	2.3 ± 5.5	4.1 ± 6.4	0.002
Disease duration (year)	NA	10.9 ± 11.8	12.1 ± 11.4	0.583
Allergic rhinitis (%)	0	59.6	72.7	< 0.001
Chronic rhinosinusitis (%)	0	36.2	41.6	< 0.001
Nasal polyp (%)	0	8.5	14.3	0.072
ACT score	25.0 ± 0.0	20.2 ± 5.1	17.0 ± 5.0	< 0.001
ACQ score	0.0 ± 0.0	1.0 ± 0.9	2.1 ± 1.1	< 0.001
FEV ₁ (%)	100.6 ± 11.4	94.4 ± 12.7	65.5 ± 15.8	< 0.001
FEV ₁ /FVC (%)	80.9 ± 7.3	75.4 ± 6.9	64.3 ± 12.8	< 0.001
Vitamin D (ng/mL)	18.6 ± 7.5	21.4 ± 12.1	19.6 ± 11.0	0.502
Eosinophilia (%) [†]	7.7	73.3	64.9	< 0.001
Metropolitan residence (%)	81.8	83.0	64.9	0.071

Values are expressed as n (%) or mean ± SD

Abbreviations: BMI, body mass index; SABA, short-acting beta agonists; ACT, asthma control test; ACQ, asthma control questionnaire; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity

*Fisher's exact test for categorical variables, Kruskal-Wallis test with Tukey's post-hoc analysis for continuous variables

[†]Eosinophilia was defined as either baseline blood ≥ 300 cells/ μ L or sputum eosinophils $\geq 3\%$ at least.

Table S2. Differential genera associated with non-asthma and asthmatic subjects by ANCOM method (FDR < 0.1)

Group	Method 1 (multcorr option = 3)	
	Genus	Relative Proportion
Non-asthma > Asthma*	<i>Selenomonas</i>	0.0306 ± 0.0136 vs. 0.0028 ± 0.0007
	<i>Campylobacter</i>	0.0124 ± 0.0023 vs. 0.0062 ± 0.0007
	<i>JN713389_g</i>	0.0043 ± 0.0017 vs. 0.0011 ± 0.0004
	<i>Saccharimonas</i>	0.0223 ± 0.0076 vs. 0.0081 ± 0.0018
Non-asthma > Severe	<i>Selenomonas</i>	0.0306 ± 0.0136 vs. 0.0025 ± 0.0009
	<i>Campylobacter</i>	0.0124 ± 0.0023 vs. 0.0057 ± 0.0009
Non-asthma < Asthma	<i>Alloprevotella</i>	0.0065 ± 0.0016 vs. 0.0128 ± 0.0031
	<i>Fusobacterium</i>	0.0224 ± 0.0056 vs. 0.0334 ± 0.0056
	<i>Rothia</i>	0.0303 ± 0.0095 vs. 0.0379 ± 0.0066

*Asthma includes both non-severe and severe asthma

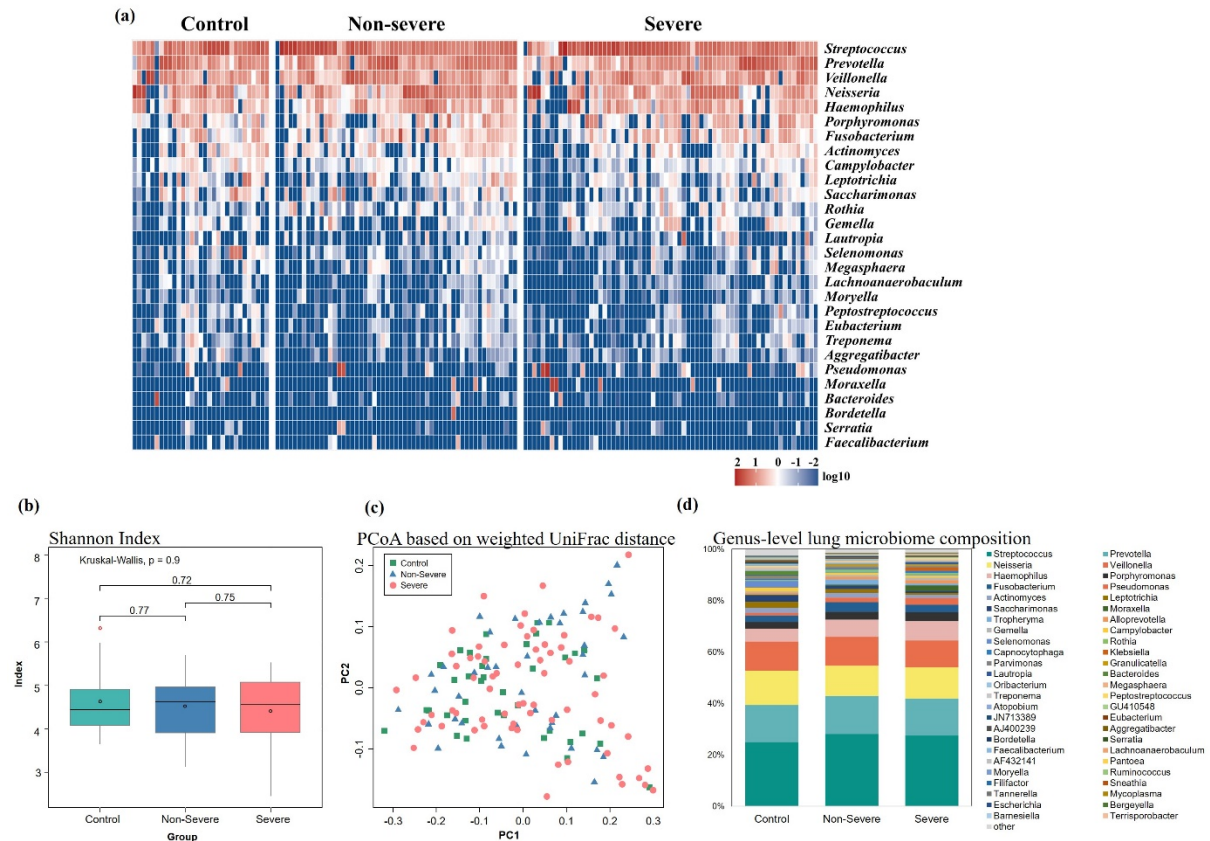


Figure S1. Comparison of microbial communities in non-asthma control, non-severe, and severe asthmatics.

(a) Heatmap demonstrating log-transformed abundances of 28 top genera among non-asthma controls, non-severe and severe asthma patients. The percentage abundance of bacterial genera was $\log_{10}$ transformed and visualized using the hclust package with the complete clustering option using R. **(b)** Comparison of α -diversity (Shannon index) among groups of non-asthma, non-severe, and severe asthmatics. **(c)** Comparison of β -diversity among groups of non-asthma, non-severe, and severe asthmatics by weighted normalized UniFrac distance matrix. **(d)** Bar graph showing the relative proportion of major genera among non-asthma control, non-severe, and severe asthmatics.

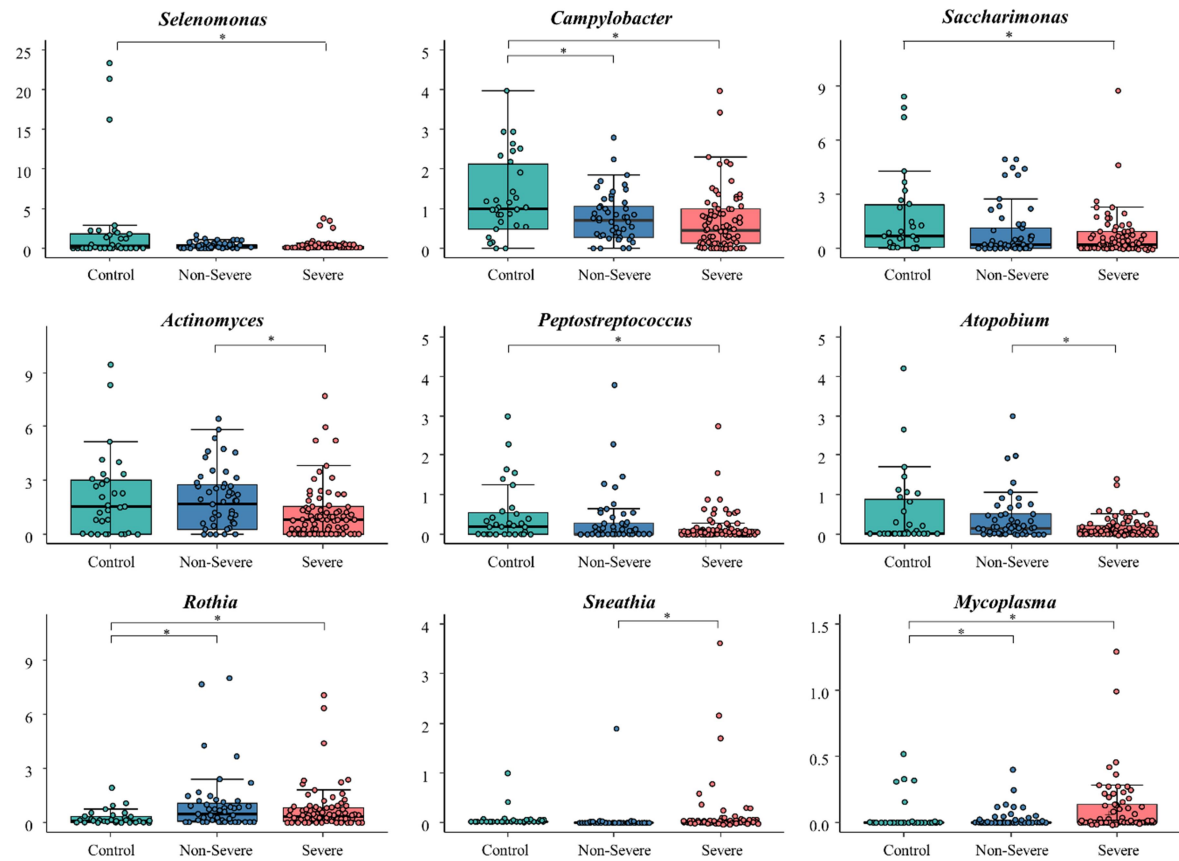


Figure S2. Relative abundance (%) of different bacterial genera in non-asthma, non-severe and severe asthma subjects.

Significant microbial genera are represented by bar graphs. One-way analysis of variance, followed by post hoc Dunn's test (error bars, mean $\pm$ standard error of mean). * $P < 0.05$ significantly different between the indicated groups.

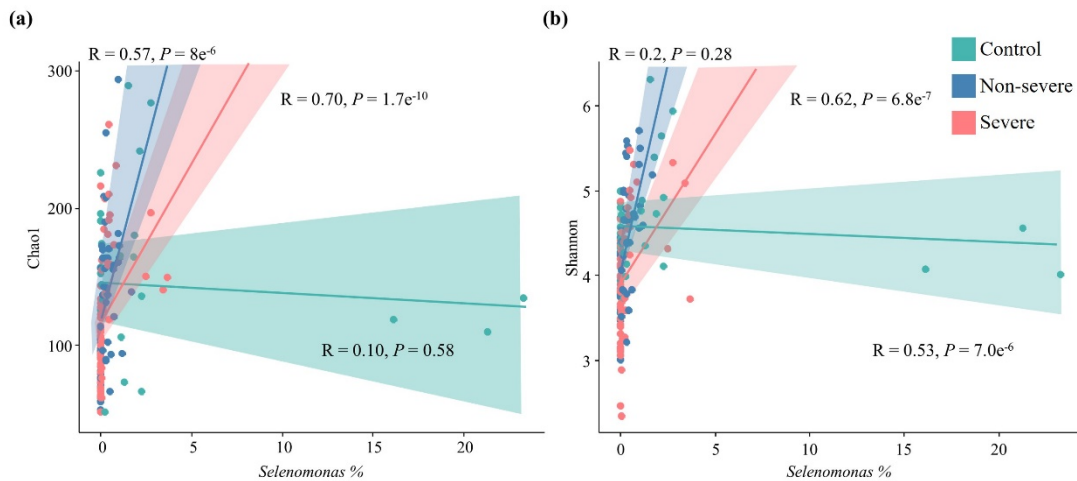
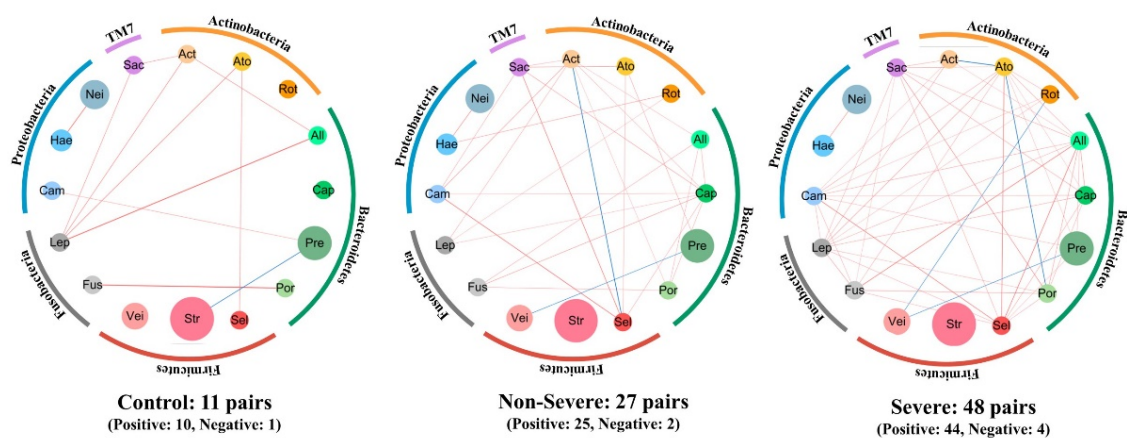


Figure S3. Correlation between the abundance of *Selenomonas* and sputum microbiome diversity indices in non-asthma, non-severe and severe asthma groups.

Spearman's rank correlation was calculated between the relative percentage of *Selenomonas* and diversity indices. Correlation between relative abundance (%) of *Selenomonas* and Chao1 index (a) or Shannon index (b). Shading areas represent 95% confidence intervals.



FDR p value < 0.1

Figure S4. Microbial co-occurrence networks for non-asthma control, non-severe and severe asthma groups.

Each node corresponds to one genus of microbiome and the color of the line shows a positive (red) or negative (blue) relationship. The line width is proportional to the correlation coefficient, and the size of the node reflects on the composition ratio (Act: *Actinomyces*, All: *Alloprevotella*, Ato: *Atopobium*, Cam: *Campylobacter*, Cap: *Capnocytophaga*, Fus: *Fusobacterium*, Hae: *Haemophilus*, Lep: *Leptotrichia*, Nei: *Neisseria*, Por: *Porphyromonas*, Pre: *Prevotella*, Rot: *Rothia*, Sac: *Saccharimonas*, Sel: *Selenomonas*, Str: *Streptococcus*, Vei: *Veillonella*).

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