

Letter to the Editor

Histamine-secreting microbes are increased in the gut of adult asthma patients

To the Editor:

Microbiome-host interactions are believed to significantly influence host immunologic homeostatic mechanisms, whereas microbial dysbiosis has been associated with many inflammatory diseases, including asthma.¹ In addition to alterations in microbiome composition, the metabolic activity of the microbiome is relevant.² One such metabolite is histamine, which is a biogenic amine with multiple effects on immunoregulatory and effector responses.³ Secretion of histamine by microbes significantly influences immune responses within the gut, which is mediated in part by histamine type 2 receptor.⁴⁻⁶ Evaluation of histamine secretion by the human gut microbiome has not been previously assessed in healthy human volunteers or patients with asthma. In the present study, we quantified the burden of histamine-secreting bacteria in the human gut microbiome and identified specific bacterial strains that secrete high levels of histamine *ex vivo*.

Fecal samples were obtained from healthy volunteers (n = 74; mean age, 41.7 years; male:female, 29:45) and adult patients with asthma (n = 74; mean age, 41.9 years; male:female, 29:45). The study-specific procedures were approved by the Wrocław Medical University Ethics Committee. The bacterial histidine decarboxylase (HDC) gene was quantified in isolated DNA using primers validated for bacterial HDC, but that do not amplify the human HDC gene (for a representative gel, see Fig E1 in this article's Online Repository at www.jacionline.org). Twelve different primer pairs (see Table E1 in this article's Online Repository at www.jacionline.org) were used for quantification of the bacterial HDC gene because no one primer set can amplify all bacterial HDC gene sequences that have been previously described. Bacterial HDC gene levels were significantly elevated in adult patients with asthma compared with healthy controls (Fig 1, A). Within the asthma group, there were clearly patients with high or low HDC levels. To determine the patient characteristics that might better identify patients with high or low bacterial HDC levels, we separated patients on the basis of demographic and available clinical data. Interestingly, the patient characteristic that gave the best separation was body mass index (BMI). Nonobese patients with asthma (n = 39; BMI, 20-25) had the highest level of the bacterial HDC gene (Fig 1, B) compared with obese patients with asthma (n = 35; BMI, >30). There was no difference in bacterial HDC levels between nonobese (n = 37) and obese (n = 37) controls without asthma (Fig 1, B). As a possible explanation for the difference in bacterial HDC levels between obese and nonobese patients with asthma, we assessed dietary habits using a food frequency questionnaire, but no significant differences were observed between the different groups (see Table E2 in this article's Online Repository at www.jacionline.org). However, more detailed dietary analysis is required before definitive conclusions can be made on the influence of diet on histamine-secreting microbes within the gut.

We further selected those patients with asthma with the highest HDC level using the 90% percentile (>387 HDC gene copy number/ng DNA, termed HDC^{high}) and compared them with all

remaining patients with asthma (<387 HDC gene copy number/ng DNA, termed HDC^{low}). HDC^{high} patients had significantly lower forced expiratory volume (FEV₁) values compared with HDC^{low} patients (see Table E3 in this article's Online Repository at www.jacionline.org). All HDC^{high} patients (100%) were atopic (to house dust mite and/or grass pollen allergens), but most HDC^{low} patients (76%) were also atopic, suggesting that atopy is not caused by a high burden of histamine-secreting bacteria, but the presence of a large number of these bacteria may support an allergic phenotype (see Table E3). Other bacterial metabolites generated in the gut (eg, short-chain fatty acids) have clearly been shown to influence susceptibility and severity of allergic disorders.^{7,8} Similarly to histamine, short-chain fatty acids exert many of their effects by binding to G protein-coupled receptors and perhaps there are additional immunoregulatory G protein-coupled receptors, yet undiscovered, that respond to bacterial-derived metabolites. None of the other parameters assessed (age, sex, smoking or alcohol history, drug usage, or severity indices) were significantly different between HDC^{high} and HDC^{low} patients with asthma (see Table E3).

To identify some of the bacterial strains, within the gut microbiome of HDC^{high} patients with asthma, that secrete histamine, we cultured their fecal samples on multiple growth media, under multiple different growth conditions, in the presence of histidine and a pH color change indicator (protocol details are included in this article's Online Repository at www.jacionline.org). Presumptive histamine-secreting strains were further screened using quantitative PCR and ultra-performance liquid chromatography to confirm the presence of the HDC gene and the secretion of histamine, respectively (for a representative ultra-performance liquid chromatography chromatogram, see Fig E2, A, in this article's Online Repository at www.jacionline.org). Finally, bacterial culture supernatants were used to stimulate CHO cells, which possess a histamine receptor 1 (HR1) chemiluminescent reporter. Histamine in the bacterial culture supernatants activated the HR1 on these cells and activity was blocked using the HR1 antagonist diphenhydramine (see Fig E2, B). A number of bacterial isolates that secrete histamine have been identified from HDC^{high} patients with asthma, and 16S sequencing of these strains revealed that most are *Escherichia coli*, with *Lactobacillus vaginalis* and *Morganella morganii* strains also being identified as histamine-secreting organisms (Table 1). Interestingly, both *E coli* and *M morganii* are frequently implicated as causative agents in scombroid food poisoning (a histamine-mediated pathology).^{6,9} Using 16S primers specific to either *E coli* or *M morganii*, we quantified the presence of these microbes in the fecal samples of healthy volunteers and patients with asthma from our cohort. *E coli* levels were not significantly different between the groups (see Fig E3 in this article's Online Repository at www.jacionline.org), but *M morganii* levels were significantly elevated in the gut microbiome of nonobese patients with asthma, compared with obese patients with asthma, obese patients without asthma, or nonobese controls without asthma (Fig 1, C). Within the nonobese asthma group, patients with more severe disease had the highest levels of *M morganii* (Fig 1, D). Disease severity (mild, moderate, or severe asthma) was defined by medication use, medication dose, and a symptom control evaluation.

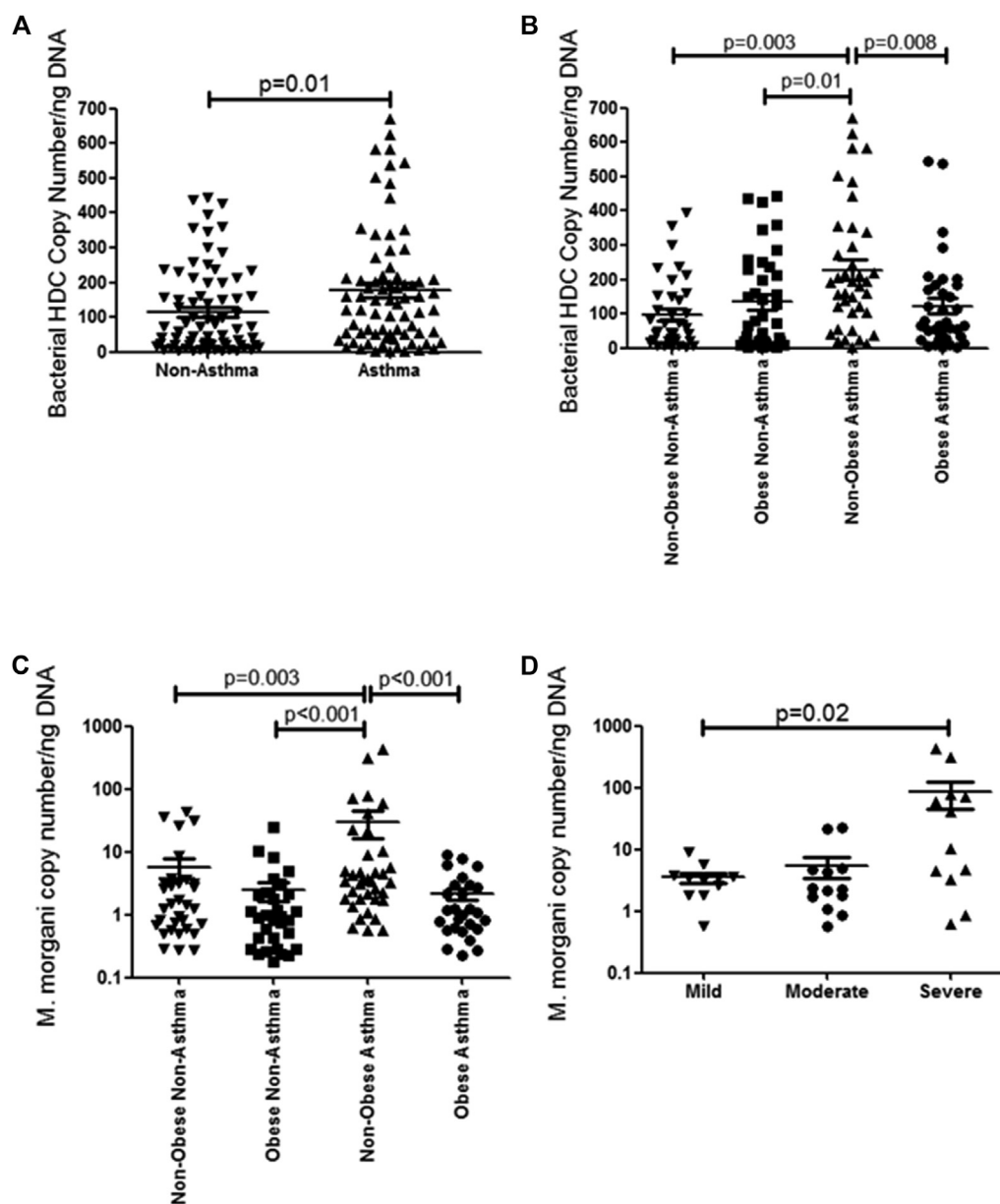


FIG 1. HDC+ bacteria are increased in the gut of adult patients with asthma. **A**, Quantitative PCR assessment of fecal samples from healthy volunteers and patients with asthma revealed that the number of HDC-positive bacteria are significantly increased in the gut microbiome of patients with asthma. **B**, Within the asthma group, nonobese (BMI, <25) patients with asthma displayed the highest level of HDC-positive microbes, compared with obese (BMI, >30) patients with asthma. Bacterial HDC levels in nonobese patients with asthma were also significantly increased compared with nonobese and obese volunteers without asthma. **C**, *Morganella morganii* levels are significantly increased in nonobese patients compared with all other groups and *M morganii* levels correlate with disease severity (**D**).

Although culture-dependent approaches are very biased because most intestinal microbes cannot be cultured yet *in vitro*, these results suggest that histamine-producing microbes, particularly *M morganii*, should be examined in more detail in the context of asthmatic diseases.

No longer can we assume that mast cells and basophils are the principal cellular sources of histamine in the human body because clearly many resident bacterial strains produce histamine and their levels are increased in the gut of adult nonobese patients with asthma. On the basis of these observations, we speculate that

increased levels of bacterial-derived histamine in certain adult patients with asthma may contribute to histamine-mediated pathologies due to a higher systemic level of histamine, which then reduces the level required for host-derived histamine to drive allergic responses following allergen exposure. The difference in HDC-positive bacteria between obese and nonobese patients with asthma suggests that factors other than histamine secretion by microbes in the gut may be more important for the development of asthma in obese patients. Indeed, this report adds to the growing body of evidence that asthma in obese individuals represents a

TABLE I. Histamine secretion by isolated bacterial strains

Strain	Histamine (mg/L)*			16S rRNA GenBank accession no.
	24 h	48 h	96 h	
<i>Escherichia coli</i>				
1DT	242	381	521	KU612261
2DT	12.8	43.3	179	KU612262
3DT	261	363	722	KU612263
4DT	0	197	591	KU612264
5DT	17.4	14.3	182	KU612265
<i>Morganella morganii</i>				
6DT	6845	6776	7722	KU612266
<i>Lactobacillus vaginalis</i>				
7DT	8870	7029	7961	KU612267
<i>Enterobacter aerogenes</i>	7548	7676	6019	—
ATCC 13048†				
<i>Lactobacillus casei</i>	0	0	0	—
ATCC 334‡				
<i>Enterococcus durans</i>	0	0	0	—
ATCC 6056‡				

ATCC, American Type Culture Collection.

*Histamine was detected via ultra-performance liquid chromatography after 24-, 48-, and 96-hours incubation in Brain Heart Infusion agar supplemented with 1% histidine and 0.005% pyridoxal-HCl (pH 6.5) at 37°C.

†Positive control strain for histamine secretion.

‡Negative control strains for histamine secretion.

distinct endotype. More accurate endotyping of patients with asthma may be assisted by further analysis of the composition and metabolic activity of an individual's microbiome, and future clinical studies of new therapeutic agents should consider performing microbiome and metabolite analyses to determine whether specific microbiome features correlate with responses to treatment. In addition, therapeutics directly targeting microbiome activities may be considered as complementary to existing drugs.

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METHODS

Quantification of HDC genes from fecal samples using quantitative PCR

Fecal samples were obtained from all individuals early in the day and were transported at 4°C to the clinic. All samples were frozen at −80°C within 4 hours of collection. DNA was isolated from the fecal samples with QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) and all the samples were adjusted to the same concentration. Following DNA isolation, bacterial HDC was quantified using quantitative PCR (qPCR). Because of the high level of polymorphisms of the bacterial HDC gene, 12 different primer sets were used to investigate the presence of HDC genes (see Table E1). qPCR analysis was performed by using an Applied Biosystems 7900 HT Fast Real-Time PCR system. The cycling conditions were as follows: 40 cycles of 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute.

Isolation of histamine-secreting bacteria

Bacteria were isolated from fecal samples from patients with asthma. Two milliliter of PBS (Life Technologies Corporation, Grand Island, NY) was added to each fecal sample, and the obtained suspension was vortexed until homogeneity. The solution was centrifuged at 4°C, 1000 rpm, for 3 minutes to pellet the fecal material. Hundred microliter of supernatant was plated on Brain Heart Infusion agar (BHI; Oxoid Ltd, Basingstoke, Hampshire, England), Tryptic Soy Agar (Oxoid Ltd), de Man, Rogosa, and Sharpe agar (Becton, Dickinson and Company, Sparks, Md), and Reinforced Clostridial Medium agar (Oxoid Ltd) supplemented with 2.7% histidine, and 0.006% bromocresol purple (Acros Organics, Morris Plains, NJ). Bromocresol purple was added to the medium as a pH indicator, because when bacteria produce histamine, the pH increases and the colony can be identified because of its purple color. Pure isolates were obtained by transferring single colonies 3 times onto the corresponding medium, and were then cryopreserved in 40% glycerol (Sigma-Aldrich, St Louis, Mo). Because bacterial histamine production depends on the growth conditions, each purified isolate was then screened under 12 different conditions in a 96-well format: 4 media (BHI, Reinforced Clostridial Medium agar, de Man, Rogosa, and Sharpe agar, and Tryptone Soya Broth) and 3 different pHs (6.5, 6.0, and 5.5) were used. Media were supplemented with 0.005% of pyridoxal-5-phosphate, 0.006% bromocresol purple, and 1% histidine. Plates were incubated at 37°C under anaerobic conditions. Histamine production was determined visually after 24 and 48 hours, and was considered as positive when medium color turned purple under at least 1 condition after 48 hours.

Quantification of HDC genes from isolates via qPCR

A total of 161 presumptive isolates were obtained from 14 fecal samples from patients with asthma and were screened for the presence of the HDC gene using qPCR under the same conditions and with the same primers as described above.

Identification of histamine-producing bacteria by 16S sequencing

Genomic DNA was purified from the isolates using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). The 16S rRNA genes were amplified via PCR using primers 27F and 1492R and GoTaq Green master mix polymerase (Promega Corporation, Madison, Wis) under the following conditions: (1) 95°C for 1 minute; (2) 95°C for 40 seconds, 54°C for 45 seconds, and 72°C for 1 minute (32 cycles); and (3) 72°C for 10 minutes. The 16S rRNA genes were sequenced by Microsynth AG (Balgach, Switzerland) and subsequently compared with all GenBank entries by BLASTN search. Sequences were uploaded to GenBank and assigned a GenBank accession number (Table I).

Quantification of histamine secretion by ultra-performance liquid chromatography

Standard and samples preparation. Standard chemicals for histamine dihydrochloride, putrescine dihydrochloride, and cadaverine dihydrochloride (Acros Organics) were of analytical grade. Standard stock solutions were separately prepared at 2000 mg/L in distilled water, and were further diluted for experiments.

Isolated bacteria were grown in BHI medium supplemented with pyridoxal-5-phosphate at 37°C and under anaerobic conditions as described above. Bacterial supernatants were obtained after 24, 48, and 96 hours by centrifugation at 9000g for 10 minutes. Samples were stored at −20°C until derivatization.

Dansyl chloride derivatization. One milliliter of bacterial supernatant or standard solution was mixed with 200 µL of 2 mol NaOH, 300 µL of saturated sodium bicarbonate solution, and 2 mL of dansyl chloride (Sigma-Aldrich) solution (10 mg/mL in acetone), and was then incubated at 40°C for 45 minutes. Residual dansyl chloride was removed by adding 100 µL of 25% ammonium hydroxide (Merck, Darmstadt, Germany). After 30 minutes at 25°C, the volume was adjusted to 5 mL with acetonitrile (Biosolve Chimie, Dieuze, France), centrifuged at 3000g for 5 minutes, and supernatants were filtered (0.22 µm) before ultra-performance liquid chromatography (UPLC) analysis.

Apparatus and separation conditions. The separation was carried out on an ACQUITY UPLC H-Class Bio System (Waters Corp, Milford, Mass) equipped with a quaternary solvent manager, sample manager FTN, column manager, and diode array detector. Data processing was performed using MassLynxV4.1 (Waters Corp). An ACQUITY UPLC BEHC18 column (1.7 µm particle size, 2.1 mm × 50 mm, Waters Corp) was used for the separation of biogenic amines, and the samples were eluted with a gradient elution of (A) acetonitrile (100%), (B) acetonitrile (50%) as follows: 0–2 minutes, A 30%, B 70%; 2–4 minutes, A 30% to 45%, B 70% to 55%; 4–5.7 minutes, A 45% to 90%, B 55% to 10%; 5.7–7.7 minutes, A 90% to 95%, B 10% to 5%; 7.7–8.7 minutes, A 95% to 30%, B 5% to 70%; 8.7–13.7 minutes, A 30%, B 70%. The flow rate was kept at 0.22 mL/min, column temperature at 25°C, injection at 1 µL, and the detection wavelength was 217 nm.

Histamine receptor signaling

The PathHunter CHO-K1 histamine receptor 1 (H₁R) β-arrestin cell line (DiscoverX, Fremont, Calif) was used to assess whether histamine secreted by bacteria would activate H₁R *in vitro*. Activation of the histamine receptor in this cell line induces β-Arrestin-EA recruitment, forcing complementation of 2 enzyme fragments. The resulting functional enzyme hydrolyzes a substrate to generate a chemiluminescent signal. Dilutions of filtered bacterial culture supernatants were added to the cells in the presence or absence of the H₁R antagonist diphenhydramine (Sigma-Aldrich). Histamine (Sigma-Aldrich) was used as a positive control. Cell cultures and reporter measurements were performed according to the manufacturer's instructions (DiscoverX).

Statistical analysis

Statistical significance between groups was calculated using the parametric *t* test or the nonparametric *t* test Mann-Whitney as appropriate. Differences in proportions were calculated using chi-square tests. All data analysis was carried out using GraphPrism software (GraphPad Software, Inc, San Diego, Calif).

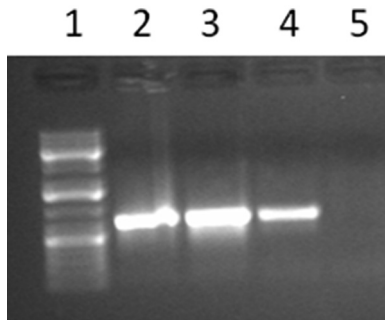


FIG E1. A representative gel demonstrating amplification of bacterial, but not human, HDC. A single band was observed following PCR on human fecal samples using the HDC primers HDC3_HDC4 (lanes 2-4). However, no amplification was observed when human DNA was used instead of fecal-derived DNA (HDC3_HDC4 primers in lane 5).

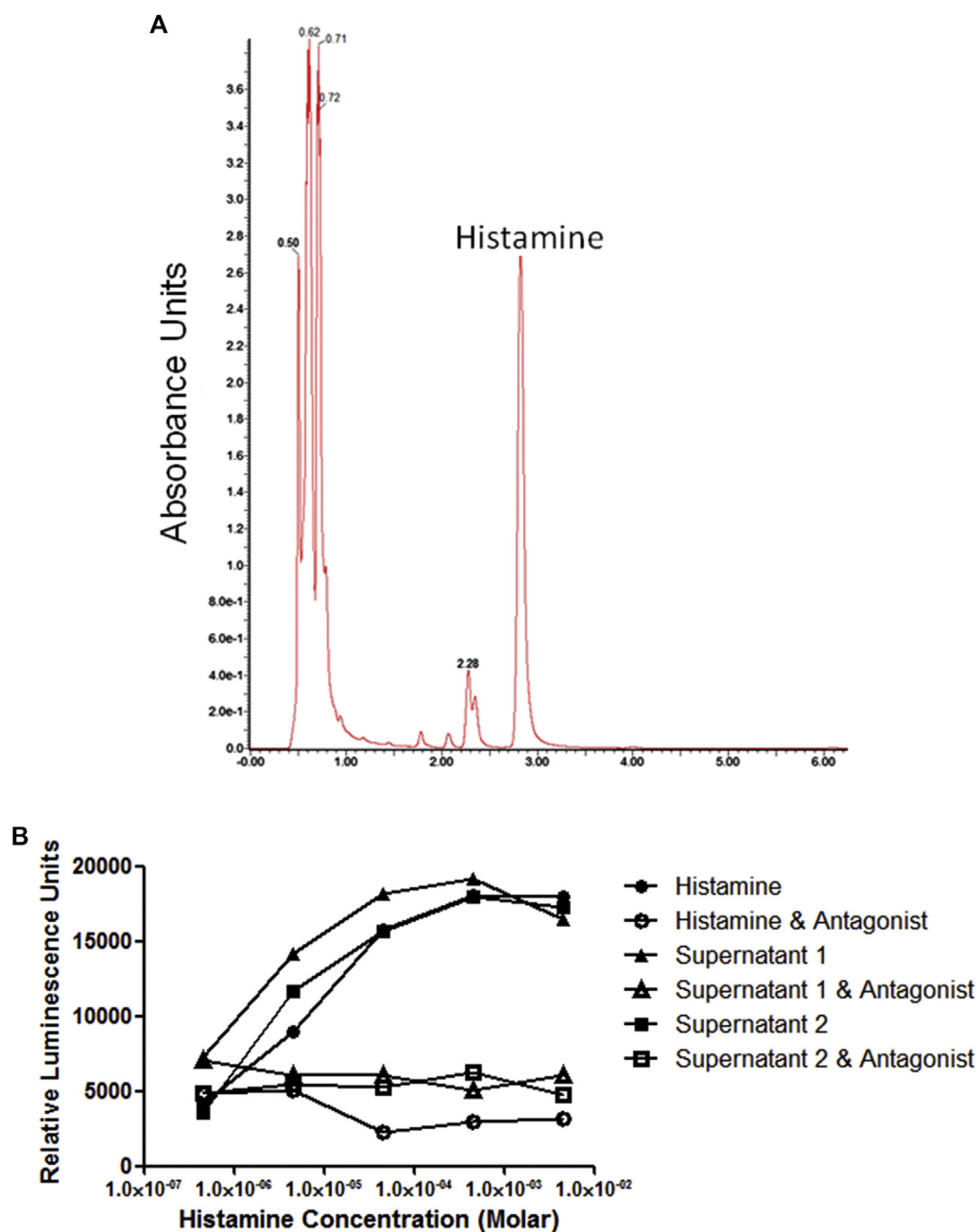


FIG E2. Histamine in bacterial culture supernatants. **A**, A representative UPLC chromatogram illustrating the detection of bacterial-derived histamine in culture supernatants. **B**, Bacterial culture supernatants, containing histamine, activate histamine receptor 1, on a reporter cell line, which is blocked by the histamine receptor 1 antagonist diphenhydramine.

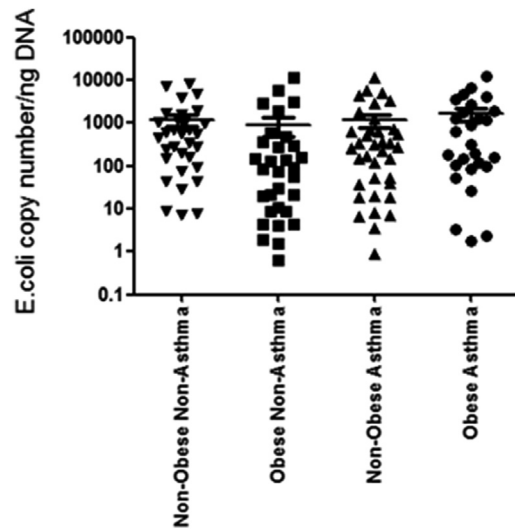


FIG E3. *Escherichia coli* levels in fecal samples. *E. coli* are present at similar levels within the gut microbiome of patients with asthma and volunteers without asthma.

TABLE E1. Primer sequences used for quantitative real-time PCR analysis

Primer set	Sense primer	Antisense primer
CL1_ CL2	CCWGGWAAWATWGGWAATGGWTA	GAWGCWGTWGTGCATATTWATTTGWCC
JV16HC_ JV17HC	AGATGGTATTGTTTCTTATG	AGACCATACACCATAACCTT
JV17_ CL1mod	AGACCATACACCATAACCTTG	CCAGGWAACATTGGTAATGGATA
HDC3_ HDC4	GATGGTATTGTTTCKTATGA	CAAACACCAGCATCTTC
HIS1-F_ HIS1-R	GGNATNGTNWSNTAYGAYMGNGCNGA	ATNGCDATNGCNSWCCANACNCCRTA
Hdc1_ Hdc2	TTGACCGTATCTCAGTGAGTCCAT	ACGGTCATACGAAACAATACCATC
HDC1_ HDC2	ATGTCAGAGTTTGATAAAAAG	TTAATAATTGATGTTTCCACC
HDC-for_ HDC-rev	TGGTATTGTTTCGTATGACCG	GGCTTCATCATTCATGTGTC
KPF2_ KPR4	AAAGCTGGGGGTATGTGACC	GTGATGGAGTTTTTGTTCG
106_107	AAYTCNTTYGAYTTYGARAARGARG	ATNGGNGANCCDATCATYTTRTGNCC
H1S2-F_ H1S2-R	AAYTSNTTYGAYTTYGARAARGARGT	TANGGNSANCCDATCATYTTRTGNCC
hdc-f_ hdc-r	TCHATYARYAACTGYGGTGACTGGRG	CCCACAKCATBARWGGDGTRTGRCC
Mm453F- Mm631R (<i>M. morganii</i>)	TTTCAGTCGGGAGGAAGGTG	GGGGATTTACATCTGACTC
EC23S857 F – R (<i>Escherichia coli</i>)	GGTAGAGCACTGTTTTGGCA	TGTCTCCCGTGATAACTTTCTC

TABLE E2. Consumption frequency of major food groups (number of times per week)

Food group	Nonobese nonasthma	Obese nonasthma	Nonobese asthma	Obese asthma	<i>P</i> value
Vegetables	4.9 ± 1.9	4.8 ± 1.7	5.9 ± 3.3	5.4 ± 2.3	.148
Meat & meat products	4.1 ± 2.0	4.8 ± 3.0	5.0 ± 2.2	5.0 ± 3.9	.501
Fish & fish products	1.7 ± 1.4	1.9 ± 1.5	1.6 ± 1.2	1.7 ± 1.4	.722
Fruits & fruit products	5.4 ± 2.2	5.4 ± 1.9	5.5 ± 3.2	5.3 ± 2.7	.996
Salads	4.3 ± 1.6	4.0 ± 2.0	4.2 ± 2.1	4.1 ± 2.6	.909

Values are mean ± SD.

TABLE E3. Demographic and clinical characteristics of HDC^{high} and HDC^{low} patients with asthma and controls without asthma

Characteristic	HDC ^{high}	HDC ^{low}	Controls	<i>P</i> value
n	9	65	74	
BMI	26.7 ± 6.6	28.9 ± 7.3	29.1 ± 7.8	.686
Age (y)	39.3 ± 11.5	42.2 ± 12.1	41.7 ± 13.3	.809
Male/female	3/6	26/39	29/45	.929
Smoker	1 (11)	12 (18)	28 (38)	.023
Alcohol (U/wk)	2.0 ± 2.4	2.0 ± 3.4	2.1 ± 2.4	.969
FEV ₁ %	65.8 ± 14.4	75.9 ± 9.5	NA	.032
Atopic	9 (100)	50 (76)	3 (4)*	.001
Antibiotics	1 (11)	4 (6)	1 (1)	.194
Daily ICS	6 (66)	57 (87)	NA	.097
Daily ICS dose†	768 ± 674	810 ± 681	NA	.951
Systemic steroids	2 (22)	6 (9)	NA	.239
SABA	6 (66)	35 (54)	NA	.468
LABA	5 (56)	43 (66)	NA	.390
Daily LABA dose‡	67 ± 70	73 ± 71	NA	.794
Antihistamines§	6 (66)	39 (60)	NA	.701
Leukotriene receptor antagonist	6 (66)	36 (55)	NA	.522
Daytime asthma symptoms >2 times?	2 (22)	20 (31)	NA	.559
Exercise/activity limited because of asthma?	5 (55)	29 (44)	NA	.537
Waking at night due to asthma symptoms?	4 (44)	21 (32)	NA	.471
Use of rescue medications >2 times?	3 (33)	19 (29)	NA	.801

Values are mean ± SD or n (%). Statistically significant differences are highlighted in boldface (*P* < .05).

ICS, Inhaled corticosteroid; LABA, long-acting beta agonist; NA, not available/applicable; SABA, short-acting beta agonist.

*Food allergy or atopic dermatitis.

†Dose of inhaled glucocorticosteroids calculated as a budesonide equivalent µg/d.

‡Dose of LABA calculated as a salmeterol equivalent µg/d.

§Antihistamine receptor 1 antagonists.