Indoor bacteria and asthma in adults: a multicentre case–control study within ECRHS II

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ABSTRACT Both protective and adverse effects of indoor microbial exposure on asthma have been reported, but mostly in children. To date, no study in adults has used non-targeted methods for detection of indoor bacteria followed by quantitative confirmation.

A cross-sectional study of 198 asthmatic and 199 controls was conducted within the European Community Respiratory Health Survey (ECRHS) II. DNA was extracted from mattress dust for bacterial analysis using denaturing gradient gel electrophoresis (DGGE). Selected bands were sequenced and associations with asthma confirmed with four quantitative PCR (qPCR) assays.

15 out of 37 bands detected with DGGE, which had at least a suggestive association ($p<0.25$) with asthma, were sequenced. Of the four targeted qPCRs, *Clostridium* cluster XI confirmed the protective association with asthma. The association was dose dependent (aOR 0.43 (95% CI 0.22–0.84) for the fourth *versus* first quartile, p for trend 0.009) and independent of other microbial markers. Few significant associations were observed for the three other qPCRs used.

In this large international study, the level of *Clostridium* cluster XI was independently associated with a lower risk of prevalent asthma. Results suggest the importance of environmental bacteria also in adult asthma, but need to be confirmed in future studies.

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Introduction

Current evidence suggests the importance of the human microbiome, both in the gut and in the airways, in the development of the immune system and in the protection from systemic disease, including asthma and allergies [1, 2]. The human microbiota originates from our environment, which has changed dramatically as a result of urbanisation. This makes the changing environmental microbiome a promising candidate for explaining the rise in the prevalence of asthma and allergic diseases [1].

It is well established that environmental microbial exposures can cause adverse respiratory effects. In addition to specific microbial pathogens causing respiratory infections, these effects include respiratory irritation and inflammation and development of asthma from endotoxin and fungal exposure in occupational setting [3] or in moisture damaged dwelling [4]. On the other hand, children living on farms with live stock or in homes with dogs [1, 2] have a lower risk of asthma and allergies than other children, possibly because of qualitative and quantitative differences in exposure to environmental microbes indoors.

The characteristics of environmental microbiota that determine if exposure results in adverse or protective health effects are still largely unknown [1]. Recent studies have emphasised the importance of microbial diversity [5–7], but the amount and type of microbial exposure, as well as possibly source and viability are still likely to be of major importance [8, 9].

Here we report on the association between indoor bacterial exposures in mattress dust and prevalent asthma in a large, international case–control study done within the adult ECRHS II population. We used denaturing gradient gel electrophoresis (DGGE) as a non-targeted DNA fingerprinting method to detect the most abundant bacterial groups in house dust samples and developed qPCR assays for quantitative confirmation of the findings. This is the first study on adult asthma using non-targeted methods for detection of the relevant bacterial exposures together with quantitative confirmation.

Materials and methods

Study design

The European Community Respiratory Health Survey (ECRHS) was initiated as a multicentre cross-sectional study on asthma and allergy in adults [10]. Later the participants were followed up as a cohort in ECRHS II at age 29–55, where 22 centres agreed to take part (for full protocols, see http://www.ecrhs.org). Written informed consent was obtained from all participants. The study was approved by the local ethics committees in each region: Reykjavík, Iceland (The National Bioethics Committee, Reykjavík, Iceland); Umea, Uppsala, Gothenburg, Sweden (Regional Ethical Committee in Uppsala, Sweden); Erfurt, Hamburg, Germany (Ethic Committee of the Bavarian State Chamber of Physicians, Germany); Norwich, UK (Norwich District Ethics Committee); Ipswich, UK (Ipswich–East Suffolk Local Research Ethics Committee); Grenoble, France (Ethics committee Paris Bichat-Claude Bernard); Barcelona, Spain (Comité Ético de Investigación Clínica del Instituto Municipal de Asistencia Sanitaria, Barcelona, Spain); Albacete, Spain (Comité de Ética e Investigación de Complejo Hospitalario de Albacete, Spain); Oviedo, Spain (Comité Ético de Investigación Clínica Regional, Hospital Universitario Central de Asturias, Oviedo, Spain); Galdakao, Spain (Comité Ético de Investigación del Hospital de Galdakao, Spain); and Basel, Switzerland (Swiss Academy of Medical Sciences and the ethics committee of Basel).

The present study population was chosen from the ECRHS II using a case–control design with frequency matching for centre; 198 current asthmatics from centres located in different climatic regions in Europe and 199 controls were selected (table 1) and mattress dust samples from 199 asthmatics and 198 controls were analysed for microbial content. The case selection was based on the following criteria: Yes to "ever asthma" in ECRHS II and Yes to "physician diagnosis" and Yes to "wheeze" or "chest tightness" or "attacks of breathlessness following activity or at rest" or "asthma attack" or "current medication" or "woken by attack of shortness of breath" in the last 12 months. Controls were defined as persons, who answered no to "ever asthma" in ECRHS II and no to "physician diagnosis" and no to "wheeze" and "chest tightness" and "attacks of breathlessness following activity or at rest" and "asthma attack" and "current medication" and "woken by attack of shortness of breath" in the last 12 months.

Dust samples

Mattress dust samples were collected during home visits in ECRHS II as described earlier [11]. Briefly, dust samples were collected from 80 cm×125 cm area of the bed where the participant usually slept. An ALK dust collection filter (ALK-Abello, Hørsholm, Denmark) was attached to an Electrolux Mondo vacuum cleaner (1300 W), and the area was vacuumed for 2 min. The dust was stored frozen at −20°C in the Imperial College, London, and for this study an aliquot was sent under dry ice for analyses at National Institute for Health and Welfare, Kuopio, Finland.

Microbial analyses

Mattress dust samples of about 20 mg were first accurately weighted and DNA then extracted using bead-beating and internal standard, as described earlier [12, 13]. For DGGE analyses, an approximately

TABLE 1 Characteristics of the cases and controls

	Cases	Controls
Parental allergy %	46.0	35.2
Smoking status %		
Lifetime non-smoker	42.4	41.7
Ex-smoker	35.4	41.2
Current smoker	22.2	17.1
Atopy %	63.3	17.8
BHR slope %		
6.01<	59.2	13.5
6.02–8.24	24.2	40.4
>8.25	16.7	46.2
Household density %		
<0.75 people per room	47.7	49.2
Male %	47.0	47.7
Age years %		
<38.8	28.3	22.1
38.8–45.0	22.7	27.2
45.1–49.9	24.8	25.1
>49.9	24.2	25.6
Subjects per centre n		
Hamburg (Germany)	11	11
Erfurt (Germany)	13	13
Barcelona (Spain)	15	15
Galdakao (Spain)	14	14
Albacete (Spain)	15	15
Oviedo (Spain)	15	15
Grenoble (France)	14	14
Ipswich (UK)	15	15
Norwich (UK)	14	15
Reykjavik (Iceland)	15	14
Gothenburg (Sweden)	15	15
Umea (Sweden)	14	14
Uppsala (Sweden)	13	14
Basel (Switzerland)	15	15
Total subjects	198	199

BHR: bronchial hyper-responsiveness to methacholine.

200 bp fragment of the 16S rRNA gene was amplified using universal bacterial primers, one of which contained a GC clamp [14]. The PCR products were separated with 8% (v/v) polyacrylamide gel (acrylamide:bisacrylamide 37.5:1, Sigma-Aldrich, Schnelldorf, Germany). The DGGE band patterns were visualised by SYBR Gold staining and Dark Reader transilluminator (Claire chemical research, Dolores, CO, USA). Digital images were captured with Canon PowerShot G9 and the images were analysed using the Bionumerics 4.7 software (Applied Maths, Sint-Martens-Latem, Belgium).

Selected DGGE bands were excised from the polyacrylamide gel and amplified using PCR. Sequence preprocessing including quality check and vector and primer sequence exclusion was done using Pregap and Gap4 programmes of the Staden Package [15]. The Pyrosequencing pipeline Classifier and Seqmatch tools of Ribosomal Database Project (RDP) package [16] were used to assign the sequences to bacterial taxa above species level and select reference sequences for phylogenetic analysis. Sequenced products were rerun to DGGE gel to check the purity and identify the correct position in the gel.

Assay for *Clostridium* spp. cluster XI was published earlier [17], but the forward primer was slightly modified (table S1) and the assay was re-optimised. The *Staphylococcus* (Staph) assay was published earlier [18]. Assays for the Corynebacterineae/Pseudonocardianeae group (Cory/Pseu) and *Corynebacterium amycolatum (Camy)* cluster are novel, and were developed during this project. All primers, probes and conditions of qPCR are presented in the supplementary material and table S1.

Dust samples were also analysed for cell wall markers of microbes: ergosterol, a marker of fungal biomass, 3-hydroxy fatty acids (3-OHFA) and muramic acid, markers of Gram-negative and Gram-positive bacteria, respectively [19]. Sample preparations of ergosterol and 3-OHFAs [19] and muramic acid [20] were carried out with slight modification of the earlier protocol, and were analysed by gas chromatography tandem mass spectrometry. The amount of lipopolysaccharide (LPS) was calculated as the sum of 10–16 chain length 3-OHFAs divided by four [21].

Other health outcomes

Serum specific immunoglobulin (Ig)E to house dust mite, timothy grass, cat and *Cladosporium*, as well as total IgE, was measured using the Pharmacia CAP System (Pharmacia Diagnostics, Uppsala, Sweden). Atopy was defined as at least one specific IgE level >0.35 $kU \cdot L^{-1}$ [22].

Bronchial hyper-responsiveness to methacholine (BHR) was conducted [23]. Inhalation was stopped when forced expiratory volume in 1 s (FEV_1) had dropped by 20% or after a maximum cumulative dose of methacholine of 2.0 mg. The methacholine challenge was carried out using Mefar MB3 dosimeter (Mefar srl, Bovezzo, Italy). The dose–response slope was calculated [22, 24].

The asthma score was calculated based on asthma symptoms in the past 12 months [25]. The ordinal score, which ranges from 0 to 5, counts positive responses regarding the following "yes" answers in the questionnaire: wheeze with breathlessness, chest tightness, attacks of shortness of breath at rest, shortness of breath after exercise and being woken by shortness of breath.

Statistical analyses

Analyses for categorical outcomes (asthma, atopy, wheezing) were performed using multivariate logistic regression model, for BHR using linear regression and for asthma score using negative binomial model. Models for atopy, BHR, and wheezing were weighted by inverse of the sampling fractions of the cases and the controls, calculated separately for each centre. Asthma score was analysed unweighted, adjusting for the case status (asthma).

Density of the DGGE bands was analysed in three categories (no band, below and above median density).

We repeated the analyses using dichotomised data (band detected/nondetected) but this resulted in little change in the selected bands. QPCR markers were analysed in quartiles, except qPCR for *C. amycolatum* in three categories (not detected, below and above median) due to the high prevalence of detects. Bacterial diversity was estimated as the number of DGGE bands detected.

Regression models were always adjusted for centre and the following a priori selected confounders: age, sex, parental allergy, current smoking and household density. Results on *Clostridium* cluster XI were further adjusted for years lived in current home, age of mattress, damp spots inside home, mould or mildew on any surface, having allergy proof cover on mattress, keeping a cat or dog and allowing it into the bedroom, and occupational exposure to substances associated with asthma risk [26]. These further adjustments had practically no effect on the observed effect estimates.

To study possible areal differences in the associations, centres were divided into three climatic areas: North Europe (Reykjavik, Gothenburg, Umea and Uppsala centres), Central Europe (Hamburg, Erfurt, Grenoble,

TABLE 2 DGGE bands with a suggestive association[#] (p<0.25) with prevalent asthma that were selected for sequencing together with summary of the result of the sequencing, and the qPCRs used in the subsequent confirmation analyses

Band name		aOR (95% CI)	p-value	Sequence	qPCR
L3B0_2	2nd tertile	0.831 (0.470–1.469)		*Staphylococcus haemolyticus/hominis/warneri/ Planococcus* sp. *Streptococcus intermedius/anginosus*	
	3rd tertile	1.488 (0.838–2.644)	0.2445		*Staphylococcus*
L3B1_3	2nd tertile	1.370 (0.775–2.419)		*Staphylococcus haemolyticus/hominis/warneri Propionibacterium acnes*	*Staphylococcus*
	3rd tertile	0.547 (0.299–1.000)	0.0431		
L3B10_0	2nd tertile	0.618 (0.307–1.244)		*Escherichia/Shigella* spp.	
	3rd tertile	0.646 (0.335–1.243)	0.2217		
L3B26_8	2nd tertile	1.707 (0.970–3.003)		*Faecalibacterium prausnitzii*	
	3rd tertile	1.152 (0.658–2.017)	0.1789		
L3B29_8	2nd tertile	1.459 (0.871–2.443)		*Actinomycetospora* sp.	
	3rd tertile	0.946 (0.556–1.611)	0.2424		
L3B32_5	2nd tertile	1.497 (0.863–2.595)		*Aerosphaera taetra*	
	3rd tertile	0.831 (0.484–1.427)	0.0995		
L3B42_0	2nd tertile	2.191 (1.089–4.405)		Sequencing unsuccessful	
	3rd tertile	1.768 (0.866–3.612)	0.0876		
L3B49_8	2nd tertile	2.499 (1.455–4.291)		*Corynebacterium amycolatum Corynebacterium coyleae/mucifaciens*	*Corynebacterium amycolatum* cluster
	3rd tertile	1.135 (0.667–1.933)	0.0017		
L3B51_6	2nd tertile	1.093 (0.630–1.897)		*Corynebacterium amycolatum*	*Corynebacterium amycolatum cluster*
	3rd tertile	0.596 (0.342–1.036)	0.0469		
L3B53_7	2nd tertile	2.206 (1.214–4.007)		*Corynebacterium mucifaciens/afermentans/ ureicelerivorans Staphylococcus*	*Corynebacterineae/ Pseudonocardiae* group
	3rd tertile	1.485 (0.819–2.692)	0.0299		
L3B55_7	2nd tertile	0.730 (0.408–1.306)		*Clostridium* spp.	
	3rd tertile	0.472 (0.261–0.853)	0.0382	*Clostridia cluster XI*	
L3B57_6	2nd tertile	2.399 (1.378–4.178)		*Corynebacterium afermentans Corynebacterium/Rothia* spp.	*Corynebacterineae/ Pseudonocardiae* group
	3rd tertile	2.319 (1.299–4.140)	0.0038		
L3B62_4	2nd tertile	1.704 (0.990–2.932)		*Kocuria palustris*	
	3rd tertile	1.103 (0.631–1.928)	0.1009		
L3B71_9	2nd tertile	2.197 (1.214–3.974)		*Lactococcus/Enterococcus.* spp. *Streptococcus* spp.	
	3rd tertile	1.768 (0.963–3.249)	0.0188		
L3B75_1	2nd tertile	0.791 (0.351–1.786)		*Staphylococcus aureus/epidermis/cohnii*	
	3rd tertile	0.483 (0.211–1.104)	0.2092		*Staphylococcus*

DGGE: denaturing gradient gel electrophoresis. [#]: DGGE bands were analysed in tertiles, lowest tertiles were used as reference (odds ratio=1) throughout. Adjusted odds ratios, together with p-values (df=2) for the association with asthma are shown. Adjusted for parental allergy, smoking status, household density, sex, age and centre.

Ipswich, Norwich and Basel centres) and South Europe (Barcelona, Galdakao, Albacete and Oviedo centres).

Most statistical analyses were done with SAS 9.3 software (SAS Institute Inc., Cary, NC, USA). To assess the shape of the studied association, an additive model with thin-plate regression spline was applied in the mixed generalised additive models with integrated smoothness estimation package (mgcv) [27] in R version 3.3.0.

Results

The study included 198 asthmatic and 199 controls aged 29–55 from 14 European centres (table 1). Cases had clearly more atopy, bronchial hyperreactivity, and parental allergy than controls, whereas differences in age, smoking and crowding were smaller.

In the DGGE analyses, 37 different bands were detected in the 397 mattress dust samples representing the most dominant bacterial species or groups. Three bands were excluded from further analyses because of the low number of samples with detectable bands. All other bands were detected in at least 33 samples.

TABLE 3 Distributions of different (untransformed) microbes (cells per mg dust) measured using quantitative PCR among asthmatics and non-asthmatics

	Subjects n	n<DL	Mean	Percentiles		
				25th	50th	75th
Corynebacterineae/Pseudonocardianeae group						
Asthma	198	0	110 000	19 000	48 000	120 000
Non-asthma	199	0	140 000	21 000	53 000	130 000
***Corynebacterium amycolatum* cluster**						
Asthma	198	76	2400	0	170	1800
Non-asthma	199	74	3700	0	320	2300
***Clostridium* cluster XI**						
Asthma	198	11	100	4	17	63
Non-asthma	199	9	120	6	22	89
Staphylococcus						
Asthma	198	12	270 000	52 000	140 000	320 000
Non-asthma	199	6	270 000	52 000	110 000	340 000

n<DL: number of samples below the detection limit.

The presence of 15 bands had at least a suggestive association ($p<0.25$) with asthma and were selected for sequencing (table 2).

Based on the sequencing results, four qPCRs were developed or optimised for mattress dust (table 3).

Concentrations of the Corynebacterineae/Pseudonocardianeae group and *Staphylococcus* group had a rank correlation of 0.47. All other correlations between the qPCRs were below 0.24.

Of the qPCRs developed, quantitative analysis of *Clostridium* cluster XI confirmed the protective association with asthma observed in the analyses of the DGGE bands. The association was dose dependent and statistically significant (table 4, figure 1). Figure 1 shows the association for a spline model with four degrees of freedom, but the linear model achieved the best fit (lower Akaike information criterion).

There was no evidence of effect modification by atopy ($p=0.9$ for test for interaction between atopy and *Clostridium* cluster XI) or by time lived in the current home ($p=0.6$ for interaction). There was also no

TABLE 4 Adjusted[#] association of the quartiles of specific quantitative PCR markers with prevalent asthma and atopy

	Asthma				Atopy			
Marker	**Subjects n**	**OR (95% CI)**	**p-value**	**Trend test**	**Subjects n**	**OR (95% CI)**	**p-value**	**Trend test**
***Corynebacterineae/Pseudonocardianeae* group cells·mg^{-1}**								
I ⩽19 340	98	1		0.1452	87	1		0.0105
II 19 341–49 099	97	0.98 (0.54–1.80)	0.9571		86	2.30 (0.83–6.33)	0.1085	
III 49 100–124 999	97	0.77 (0.41–1.45)	0.4229		87	1.57 (0.53–4.69)	0.4159	
IV ⩾125 000	96	0.66 (0.35–1.25)	0.2010		91	0.25 (0.09–0.74)	0.0125	
***Corynebacterium amycolatum* cluster cells·mg^{-1}**								
I 0	146	1		0.4954	129	1		0.4768
II 1–1319	121	1.00 (0.60–1.68)	0.9917		109	2.20 (0.95–5.06)	0.0652	
III ⩾1320	121	0.83 (0.49–1.40)	0.4742		113	1.28 (0.58–2.83)	0.5352	
***Clostridium* cluster XI cells·mg^{-1}**								
I ⩽5.50	97	1		0.0094	87	1		0.7244
II 5.51–18.35	96	0.78 (0.42–1.46)	0.4402		91	1.26 (0.47–3.38)	0.6523	
III 18.36–71.46	96	0.58 (0.31–1.11)	0.0990		84	0.49 (0.16–1.45)	0.1967	
IV ⩾71.47	99	0.43 (0.22–0.84)	0.0138		89	1.06 (0.38–2.96)	0.9111	
***Staphylococcaceae* group cells·mg^{-1}**								
I ⩽52 000	97	1		0.8696	89	1		0.7701
II 52 001–124 000	96	0.70 (0.38–1.30)	0.2549		88	1.56 (0.52–4.66)	0.4254	
III 124 001–318 450	97	1.12 (0.59–2.12)	0.7216		87	1.08 (0.34–3.37)	0.8996	
IV⩾318 451	98	0.82 (0.42–1.57)	0.5411		87	0.99 (0.30–3.21)	0.9810	

[#]: adjusted for parental allergy, smoking status, household density, sex, age and centre.

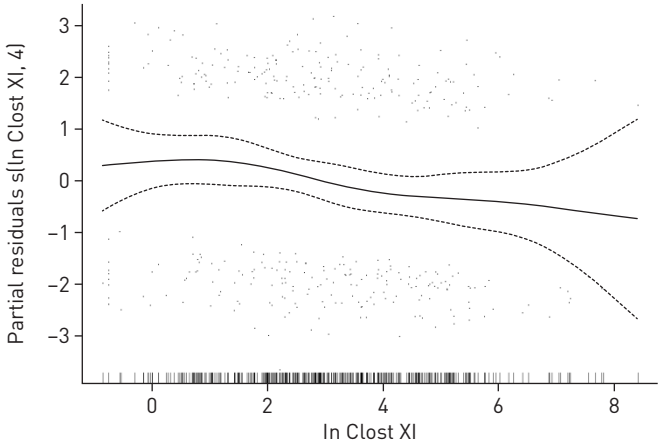

FIGURE 1 Smoothed association with four degrees of freedom between log-transformed *Clostridium* cluster XI (Clost XI) levels and the risk of asthma. The dashed curves represent the approximate 95% confidence interval. The figure is adjusted for parental allergy, smoking status, household density, sex, age and centre.

evidence that the association of *Clostridium* cluster XI with asthma would differ between North, Central and South Europe (table 5, p=0.89 for the test of interaction with area).

The observed odds ratios and p-values for asthma changed little after further adjustment for the other three bacterial groups determined with qPCRs, for bacterial diversity or for the concentrations of general bacterial and fungal biomass markers muramic acid, LPS and ergosterol (table 6). In this study microbial diversity was not associated with a risk of asthma, with adjusted odds ratio for the second tertile of 0.72 (95% CI 0.42–1.24) and for the third tertile of 1.34 (95% CI 0.78–2.29) compared with the lowest tertile.

The concentrations of *Clostridium* cluster XI were also significantly and dose-dependently inversely associated with wheezing (table S3). No clear association was observed with the other secondary outcomes, *i.e.* atopy, bronchial hyperreactivity or asthma score (table 4 and table S3).

Concentrations of the Corynebacterineae/Pseudonocardianeae group determined with qPCR were not significantly associated with asthma (table 3), but there was a tendency for a protective association (test for trend p=0.15). Although the test for interaction with area was not significant (p=0.23), the association was seen only in North Europe (table 5), where it reached statistical significance. The protective association with wheezing was significant and dose dependent (table S3). For atopy, the odds ratio was significantly below one in the highest quartile, but the association was not linear (table 3). These suggestions for protective associations for concentrations of the Corynebacterineae/Pseudonocardianeae group are somewhat in contrast with the increased risk of asthma seen in association with the most of DGGE bands, where *Corynebacterium* species were detected (table 2).

Concentrations of the *C. amycolatum* cluster were not significantly associated with asthma (table 3), but there were suggestions for increased risk of atopy (p=0.07, table 3) and BHR (p=0.07, table S3) in the middle group. Concentrations of the *Staphylococcus* group were not associated with any of the studied outcomes.

Discussion

In this first study on asthma in adults using non-targeted methods for detection of relevant indoor bacteria followed with quantitative confirmation with qPCR, *Clostridium* cluster XI had a protective association with asthma in adults. The association was highly significant, dose dependent and independent of the effect of other markers of microbial exposure. Few associations were observed for the other bacterial groups determined with qPCR, but there was some suggestion for protective associations with the Corynebacterineae/Pseudonocardianeae group.

An increasing body of evidence suggests that environmental microbial exposures, especially in early childhood and possibly during pregnancy may protect from allergic diseases [1, 2]. Most of the earlier epidemiological studies have relied on indirect markers of increased microbial exposure, such as living on a farm and having dogs in the house [2], but several studies have also measured markers of bacterial and fungal content in house dust. The results from these studies tend to support the hypothesis of protective effects of indoor microbial exposure, but the results are conflicting, possibly due to the limited number of

TABLE 5 Association of *Corynebacterineae/Pseudonocardianeae* group and *Clostridium* cluster XI specific quantitative PCR assays with asthma in three geographical regions

	North Europe				Central Europe				South Europe			
	Subjects n	**OR (95% CI)**	**p-value**	**Test for trend**	**Subjects n**	**OR (95% CI)**	**p-value**	**Test for trend**	**Subjects n**	**OR (95% CI)**	**p-value**	**Test for trend**
***Corynebacterineae/Pseudonocardianeae* group cells·mg^{-1}**												
I ⩽19 340	26	1		0.01	34	1		0.79	38	1		0.86
II 19 341– 49 099	29	0.44 (0.13–1.53)	0.20		42	2.00 (0.73–5.50)	0.18		26	0.68 (0.21–2.26)	0.53	
III 49 100– 124 999	29	0.23 (0.06–0.81)	0.02		29	1.20 (0.43–3.34)	0.73		29	1.20 (0.36–3.97)	0.76	
IV ⩾125 000	29	0.20 (0.05–0.78)	0.02		42	1.12 (0.40–3.15)	0.83		25	0.92 (0.26–3.24)	0.90	
***Clostridium* XI cells·mg^{-1}**												
I ⩽5.50	41	1		0.30	44	1		0.2	12	1		0.10
II 5.51– 18.35	20	1.63 (0.47–5.61)	0.44		49	0.78 (0.32–1.92)	0.59		27	0.27 (0.05–1.35)	0.11	
III 18.36– 71.46	23	0.72 (0.22–2.38)	0.59		38	0.71 (0.26–1.91)	0.49		35	0.25 (0.05–1.19)	0.08	
IV ⩾71.47	29	0.55 (0.16–1.86)	0.33		26	0.46 (0.15–1.43)	0.18		44	0.19 (0.04–0.97)	0.05	

TABLE 6 Multivariable[#] adjusted association of quartiles of *Clostridium* cluster XI with prevalent asthma[¶]

	Subjects n	Basic model OR	Other qPCRs OR	Microbial diversity OR	Muramic acid OR	Muramic acid, LPS, ergosterol OR
***Clostridium* cluster XI cells·mg^{-1}**						
I ⩽ 5.50	89	1	1	1	1	1
II 5.51–18.35	92	0.69	0.73	0.66	0.70	0.70
III 18.36–71.46	94	0.50	0.54	0.45	0.51	0.52
IV ⩾71.47	97	0.38	0.41	0.33	0.39	0.40
Test for trend		<0.01	0.01	<0.01	<0.01	<0.01

LPS: lipopolysaccharide. [#]: all models were adjusted for parental allergy, smoking status, household density, sex, age and centre (basic). Other models were adjusted, in addition to the basic model, for the other three qPCRs, for microbial diversity based on denaturing gradient gel electrophoresis, for muramic acid, and for muramic acid, LPS and ergosterol. [¶]: the table includes only those subjects (n=372) with non-missing data for all the variables adjusted.

markers used in these studies, differences in their specificities [1, 28] and high intercorrelations between different markers [29]. Lately many studies have focused on diversity of bacterial [5–7], or environmental [30] exposure, but amount of exposure still seems to play a role [9] and only diversity of selected bacteria may turn out to be beneficial for asthma [8]. Few studies have conducted an untargeted search for differences in microbial content of indoor dust with the aim to explore associations with asthma [7, 31–34], and the present study is to our knowledge the first one performed in adults.

We identified and then confirmed using quantitative PCR an inverse dose-dependent association between *Clostridium* cluster XI concentration in mattress dust and prevalent asthma. Clostridia are Gram-positive bacteria, which are ubiquitous in the environment. They are found in soil, sewage, marine sediment and faeces [35, 36]. *Clostridium* species are also part of the commensal gut flora and have been isolated from the female genital tract and the oral mucosa [35]. *Clostridium* cluster XIVa and IV make up a substantial part (10–40%) of the total bacterial gut microbiota and in animal studies they have been shown to promote anti-inflammatory immune responses by activating regulatory T-cells [37]. Most clostridia have a commensal relationship with the host. The only disease in which a clear causal role of a dysbiotic gut microbiota has been demonstrated is the case of *C. difficile* infections. Increased concentration of *C. difficile* in the gut was reported to be associated with caesarean delivery and increased risk of asthma in children [38]. In an earlier study in children using same microbial methodology as the present study, high levels of *Clostridium* cluster XI in mattress dust was associated with less atopy, but not asthma [39].

Although human faeces is an important source of clostridia, several lines of reasoning suggests that the observed association between *Clostridium* cluster XI and asthma in the present cross-sectional study is not related to reverse causation, *e.g.* better hygienic practices among asthmatics [40]. First, we observed little association of asthma with qPCRs for *Staphylococcus* and *Corynebacteriales*/*Pseudonocardiales*, which both have important sources from human skin and faeces. Furthermore, the association between *Clostridium* cluster XI and asthma was not affected by adjustment for other microbial markers in mattress dust, which should also be affected by better hygiene.

Clostridium cluster XI has also important environmental sources. In our earlier study [39], we observed several times higher levels of *Clostridium* cluster XI in mattress dust of children living in farming homes than those living in urban homes. This was explored further in a new exposure study with repeated dust samples of from five urban homes and four homes on active farms with livestock, where we found several times higher levels of *Clostridium* cluster XI in farming homes not only in mattress dust samples, but also in floor dust and personal air samples (unpublished data). Urban–rural difference in this study was much smaller for the Corynebacterineae/Pseudonocardianeae group.

Taken together these results suggest the importance of environmentally derived exposure to *Clostridium* cluster XI bacteria. Whether these bacteria exert their potential effect on asthma and allergies through colonisation of the gut [37] or the respiratory tract [41] or through transient irritation, needs to be determined in future studies.

As discussed above, the main limitation of our study is its cross-sectional nature. A potential methodological limitation is the limited resolution of DGGE used for bacterial fingerprinting compared with next-generation sequencing (NGS) approaches. NGS methods, or more specifically amplicon

sequencing or metagenomic shotgun sequencing, detect better microbial diversity than DGGE, and this may explain why no clear association between diversity and asthma was seen in the present study. On the other hand, fingerprinting methods, such as DGGE, display the more abundant bacterial species in a sample, which should equally be considered strength, as these more abundant taxa likely also represent the more relevant groups with respect to interference with human health. Fingerprinting methods have also been used successfully in recent studies on asthma and allergies [5–7, 34, 39]. The large number of less abundant taxa detected by NGS also complicates meaningful statistical analyses and the identification of individual, relevant exposures. Neither NGS nor DGGE are fully quantitative. This underlines the importance of confirming results using qPCRs, which is not common practice, but was done here. In general, DNA-based approaches that involve PCR, including both DGGE and amplicon sequencing, are somewhat vulnerable to biases introduced during DNA extraction or PCR amplification of the extracted DNA, which may result in preferential detection of one microbial species or group over another. However, such biases would presumably impact samples from homes of both asthmatics and non-asthmatics equally, so that there is little concern of false-positive results, while the possibility of failing to detect other relevant microbial taxa cannot be excluded.

Although the centres included in the study are all European and largely urban, a strength of the present study is that it covers a large number of centres with different types of climatic and other conditions. There was, however, no indication that the association of *Clostridium* cluster XI with asthma would differ by regions of Europe, which suggests good generalisability of the observed association to high-income urban areas. On the other hand, the present design may have insufficient power to detect microbes that have importance only in more specific areas or conditions. This was suggested by the observed significant association of the Corynebacterineae/Pseudonocardianeae group with asthma in North Europe, when no association was seen in other parts of Europe, but a test for this difference between areas failed to reach statistical significance.

In this large international case–control study of prevalent asthma in adults, *Clostridium* cluster XI was identified as potentially asthma protective bacterial group based on non-targeted search for bacteria in mattress dust. The result was confirmed using targeted quantitative PCR assay for *Clostridium* cluster XI. The association with asthma was highly significant, dose dependent and independent of the effect of other markers of microbial exposure. The results need to be repeated in prospective studies, but the findings suggest the importance of environmentally derived exposure to *Clostridium* cluster XI bacteria in asthma.

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