

Shifts in *Lachnospira* and *Clostridium* sp. in the 3-month stool microbiome are associated with preschool age asthma

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Abstract

Asthma is a chronic disease of the airways affecting one in ten children in Westernized countries. Recently, our group showed that specific bacterial genera in early life are associated with atopy and wheezing in 1-year-old children. However, little is known about the link between the early life gut microbiome and the diagnosis of asthma in preschool age children. To determine the role of the gut microbiota in preschool age asthma, children up to 4 years of age enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) study were classified as asthmatic ($n = 39$) or matched healthy controls ($n = 37$). 16S rRNA sequencing and quantitative PCR (qPCR) were used to analyse the composition of the 3-month and 1-year gut microbiome of these children. At 3 months the abundance of the genus, *Lachnospira* (L), was decreased ($P = 0.008$), whereas the abundance of the species, *Clostridium neonatale* (C), was increased ($P = 0.07$) in asthmatics. Quartile analysis of stool composition at 3-months revealed a negative association between the ratio of these two bacteria (L/C) and asthma risk by 4 years of age [quartile 1: odds ratio (OR) = 15, $P = 0.02$, CI (confidence interval) = 1.8–124.7; quartile 2: OR = 1.0, ns; quartile 3: OR = 0.37, ns]. We conclude that opposing shifts in the relative abundances of *Lachnospira* and *C. neonatale* in the first 3 months of life are associated with preschool age asthma, and that the L/C ratio may serve as a potential early life biomarker to predict asthma development.

Key words: atopic disease, dysbiosis, gut microbiota, hygiene hypothesis, microflora hypothesis.

INTRODUCTION

Asthma is a multifactorial disease driven by both genetic and environmental factors. Although there have been remarkable improvements in the treatment of asthma over the past few decades, there are currently no preventative treatments and asthma

remains the most prevalent childhood disease (affecting one-in-ten children) in many countries [1]. Multiple lines of evidence suggest that environmental factors contribute to the development of asthma, particularly the geographical disparity in disease prevalence and the observation that asthma rates have increased considerably since the 1980s—all within a single human

Abbreviations: AD, atopic dermatitis; CHILD Study, Canadian Healthy Infant Longitudinal Development Study; CI, confidence interval; FDR, false discovery rate; L/C, *Lachnospira*/*C. neonatale*; OR, odds ratio; OTU, operational taxonomic unit; qPCR, quantitative polymerase chain reaction; RQ, relative quantification.

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generation [2,3,4]. The Microflora Hypothesis suggests that early life perturbations, driven by environmental factors such as antibiotic exposure and mode of birth (vaginal vs. Caesarean section), alter the bacteria populating the intestine (i.e. cause dysbiosis) and disrupt the natural microbiota-immune cell interface critical in promoting immune tolerance [5]. Instead this dysbiosis skews the immune system toward immune-mediated and hypersensitivity disorders [5].

The intestinal microbiota has been implicated as a potential therapeutic target for the prevention of IgE-mediated hypersensitivity diseases [6–9]. Recently, our group associated early life decreases in four bacterial genera, *Faecalibacterium*, *Lachnospira*, *Veillonella* and *Rothia* (nicknamed FLVR), with atopy and wheezing in 1-year-old children enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) Study [7]. However, further research assessing the role of specific gut bacteria in the development of asthma in preschool age children is necessary before preventative treatments for this burdensome disease can be established.

Here, we describe results assessing the intestinal microbiome composition among children diagnosed with asthma by 4 years of age and control children with no history of atopy, wheezing or asthma. We show that opposing shifts in the abundance of two Clostridial taxa, *Lachnospira* and *Clostridium neonatale* (*C. neonatale*), are associated with the diagnosis of asthma by age 4 years. We quantify this gut dysbiosis by calculating the ratio of *Lachnospira*/*C. neonatale* and show an inverse correlation between this ratio in the first 3 months of life and the odds of developing asthma by 4 years of age. This ratio, in combination with the individual shifts in these two taxa in the first 100 days of life, may have important clinical implications with regard to asthma diagnosis and prevention.

MATERIALS AND METHODS

CHILD Study design and ethics approval

The CHILD Study is a longitudinal, general population birth cohort composed of 3624 families recruited at four sites across Canada (Vancouver, Edmonton, Manitoba, Toronto). The study follows infants from pregnancy to 5 years of age during which time data and biological samples related to environmental exposures, psychosocial stresses, nutrition and general health are collected. Detailed characteristics of the CHILD Study have been previously described [10–12]. Briefly, questionnaires were completed by the parents at recruitment, 36-week gestation, at 3, 6, 12, 18, 24, 30 months, and at 3, 4 and 5 years. In addition, a parent or legal guardian completes questionnaires validated in the International Study of Asthma and Allergies in Childhood (ISAAC) [13] at ages 1, 3 and 5 years. Children are also assessed at ages 1, 3 and 5 years by a CHILD Study clinician for evidence of atopic dermatitis (AD), allergic rhinitis, and asthma.

A parent or legal guardian gave signed informed consent and all research protocols for the following studies in human samples were approved by The University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board.

Subject classification

The present study is based on a nested case–control design and comprises subjects enrolled in the CHILD study that were analysed in our previous report [7]. Only children that had reached at least 3 years of age were included in this analysis (286 total subjects) and classified as follows. If a subject received a physician diagnosis of asthma by 4 years of age or was prescribed inhaled asthma medications (inhaled corticosteroids or bronchodilators) from 3 to 4 years of age, they were included in the asthmatic group ($n = 39$). To be classified as controls ($n = 37$) subjects were required to be negative for asthma or inhaled medication use, negative for atopy (based on standardized allergen skin prick testing at 1 and 3 years of age) and negative for wheezing (based on questionnaire analysis repeated six times from birth to 4 years of age combined with clinical assessments at ages 1 and 3 years).

Definitions of clinical variables

Antibiotic exposure

Continuous covariate defined by the number of oral and/or intravenous antibiotics from birth to 3 months or birth to 1-year of age.

Atopic dermatitis or eczema

'Yes' = diagnosed with AD (also referred to as eczema is a chronic skin disease characterized by itchy, inflamed skin) at 3 months (reported in 3-month CHILD health questionnaire) or at 1-year (diagnosed by a CHILD clinician at the 1-year clinical assessment or a non-CHILD clinician as reported in 1-year CHILD health questionnaire). 'No' = no diagnosis.

Feeding methods

Continuous covariate defined by the duration (in months) a child was breast fed.

Parental history of asthma

Defined as neither parent having asthma or at least one parent having asthma. Reference level is neither parent.

Delivery mode

Reference is Caesarean section birth.

Sex

Reference is female.

Microbial community analysis

Full details regarding our 16S rDNA extraction, PCR amplification and bioinformatics have been previously described [7]. Briefly, DNA was extracted from 3-month and 1-year stool samples using Mo Bio dry bead tubes (Mo Bio Laboratories), the Fastprep homogenizer (FastPrep Instrument, MP Biochemicals) or the Disruptor Genie (Scientific Industries) and the Qiagen DNA stool mini kit.

DNA samples were amplified by PCR in triplicate using bar-coded primer pairs spanning the V3 region of the 16S gene [7, 14]. V3 PCR amplicons were sequenced using Hi-Seq 2000 bidirectional Illumina sequencing (Macrogen). Sequences were quality

filtered and denoised using Mothur [15] and clustered into operational taxonomic units (OTUs) using CrunchClust [16]. Clusters were classified against the Greengenes Database [17] according to 97% similarity (Levenshtein distance = 5). OTUs with a frequency less than five among all samples were excluded.

qPCR primer design and validation

Sequences for the 16S rRNA genes of the bacterial genera and species of interest and of closely related bacteria were aligned by CLUSTAL-W using MEGA6 alignment explorer and inspected for conserved and variable regions. Based on this analysis, we designed genus-specific primer candidates for *Lachnospira* and *Rothia* and species-specific primer candidates for *C. neonatale*. Primer candidates were assessed for specificity against all bacterial sequences using Primer-Blast. The primer melting temperature, secondary structure and dimer formation, and G+C content were analysed using OligoAnalyzer3.1 (Integrated DNA Technologies). Primer pairs meeting all these requirements were validated using the standard curve method in metagenomic DNA extracted from human faecal samples (Supplementary Table S1).

Quantitative PCR conditions

Each 10 μ l reaction contained 5 μ l of IQ SYBR green supermix (Bio-Rad Laboratories), 0.1 μ l of each forward and reverse primer, 0.8 μ l of nuclease-free water and 4 μ l of faecal DNA extract. All reactions were carried out in the ViiA 7 Real-Time PCR System (Life Technologies) under the following conditions: an initial step at 95°C (5 min), 40 cycles of 15 s at 94°C, 30 s at the specific annealing temperature for each primer set (Supplementary Table S1), 30 s at 72°C (*C. neonatale*, *Veillonella* [7] and bacteria [18]) or 20 s at 72°C (*Rothia* and *Lachnospira*), and a final cycle of 95°C at 15 s, 60°C at 1 min, 95°C at 15 s and 60°C at 15 s. All samples were run in triplicate and normalized according to the ΔC_T method using total 16S rDNA (bacteria [18], Supplementary Table S1) as the reference gene. Samples with Ct values for bacteria that were two standard deviations higher than the total mean (based on all bacteria Ct values for 3 months and 1 year), indicating very low baseline levels of 16S DNA, were excluded from the analysis.

Statistical analysis

Statistical significance was defined as $P \leq 0.05$.

Logistic regression

Using the glm2 package in R, a logistic regression model was used to evaluate potential associations between the clinical variables and the asthmatic group (Table 1) [19]. Missing data were imputed with the mode of the data set for categorical variables. We report the natural log (ln) of the odds ratio (OR) and the corresponding confidence intervals (CIs). ln(OR) above 0 implies an increased likelihood that a child would develop asthma, whereas ln(OR) below 0 implies a decreased likelihood. This same model was used to confirm that all subsets of 3-month and 1-year asthmatic and control samples used in the present study were representative of the entire cohort (Supplementary Tables S2–S5).

16S sequence analysis

The microbial diversity of the faecal microbiota (based on the Shannon alpha diversity index) of asthmatics and controls was analysed in Phyloseq [20]. Deseq2 [21] was used to calculate the multi-inference adjusted (adj.) P -values (based on false discovery rate, FDR) and log₂ fold changes associated with differentially abundant OTUs between asthmatics and controls. Principal component analysis (PCA) was conducted using MetaboAnalyst [22,23].

The present study was based on a nested case–control design to study the intestinal microbiota among asthmatic and control children. La Rosa et al. report that power for microbiome analyses is associated with the number of reads per sample. *Post-hoc* power analysis of the 3-month 16S data, based on the read counts for the top 46 OTUs identified as differentially abundant by Deseq2 using the HMP R package for hypothesis testing and power calculations, resulted in a power calculation of 0.98; suggesting strong statistical power for the findings we report [24].

qPCR analyses

Differences between asthmatics and controls were assessed by the Mann–Whitney test. Differences between atopic, non-atopic asthmatics, and controls were assessed by the Kruskal–Wallis test and subject to the Dunn's multiple comparisons test. All quantitative PCR (qPCR) analyses were carried out using GraphPad Prism version 5c.

Calculation of bacterial ratios and quartile analysis

All ratios (Figure 3 and Supplementary Figure S1) were calculated by dividing the relative quantification (RQ) values (or OTU read counts normalized to relative abundance) at 3 months and 1 year. Quartiles were calculated for the ratio of *Lachnospira*/*C. neonatale* (L/C) at both time points, *Lachnospira* and *C. neonatale* individually at 3 months and 1 year. Quartiles were categorized from low (quartile 1) to high (quartile 4) to create dichotomous variables. These variables were then used to calculate ORs to determine if increases or decreases in these bacteria or ratios were associated with preschool age asthma development. ORs above 1 imply an increased likelihood of developing asthma, ORs below 1 imply a decreased likelihood.

RESULTS

Characterization of the cohort

The present study comprised 286 subjects enrolled in the CHILD Study and analysed in our previous report [7] who had reached 3 years of age at the time the present study began. Of these 286 subjects, 39 met our criteria for asthma based on physician diagnosis or having been prescribed medications used to treat asthma by 4 years of age (asthmatic group). For comparison, we identified 37 control subjects who had no evidence of asthma or allergic disease. These control subjects were negative for asthma and also negative for atopy and wheezing from birth to 3 years of age. Asthmatic and control subjects were matched for gender, birth mode (vaginal vs. Caesarean section), feeding practices (breast fed, formula fed) and antibiotic exposure

Table 1 Logistic regression analysis of key clinical variables

Variable		Phenotype		Ln(OR)	95% CI		P-value
		Asthmatics	Controls		Lower	Upper	
Antibiotic exposure (birth to 1 year of age)	1 or more	14 (36%)	5 (14%)	0.72	−0.16	1.61	0.11
	None	25 (64%)	32 (86%)				
	Total (100%)	39	37				
Antibiotic exposure (birth to 3 months of age)	1 or more	3 (8%)	2 (5%)	−1.23	−3.22	0.76	0.22
	None	36 (92%)	35 (95%)				
	Total (100%)	39	37				
AD at 3 months	Yes	7 (18%)	1 (3%)	1.13	−1.72	3.97	0.44
	No	32 (82%)	36 (97%)				
	Total (100%)	39	37				
AD at 1 year	Yes	15 (38%)	3 (8%)	1.68	0.06	3.13	0.04
	No	24 (62%)	34 (92%)				
	Total (100%)	39	37				
Sex	Female	18 (46%)	17 (46%)	−0.19	−1.3	0.91	0.73
	Male	21 (54%)	20 (54%)				
	Total (100%)	39	37				
Delivery mode	Cesarean	8 (21%)	5 (14%)	−0.16	−1.63	1.29	0.82
	Vaginal	31 (79%)	32 (86%)				
	Total (100%)	39	37				
Breast feeding	Yes	38 (97%)	34 (92%)	−0.03	−0.16	0.1	0.69
	No	1 (3%)	3 (8%)				
	Total (100%)	39	37				
Parental asthma	Neither parent	12 (31%)	26 (70%)	1.51	0.43	2.6	0.006
	At least one parent	27 (69%)	11 (30%)				
	Total (100%)	39	37				

(Table 1). In line with previous studies, children diagnosed with AD at 1 year of age or those with parental history of asthma were more likely to develop preschool age asthma [ln(OR) 1-year AD: 1.68, CI = 0.06–3.13, $P = 0.04$; ln(OR) parental history = 1.51, CI = 0.43–2.6, $P = 0.006$, Table 1] [25].

Microbial community analysis by 16S ribosomal RNA gene amplicon sequencing suggests a role for *Lachnospira* and *C. neonatale*

The global gut microbial community composition in stool samples taken at 3 months or 1 year of age did not differ between asthmatics and controls [as shown by principal components analysis and analysis of microbial diversity at 3 months and 1 year (Supplementary Figure S2)]. Beyond the analysis of global microbial community composition, we used Deseq2 with Benjamini–Hochberg adjustment (for FDR at an alpha threshold of 0.1) to identify differentially abundant OTUs between asthmatics and controls at 3 months or 1 year; with statistical significance defined as $P \leq 0.05$. At 3 months of age, five differentially abundant OTUs were identified (Figure 1A, Supplementary Table S6). Of note, OTUs 4 (*C. neonatale*, $P = 0.076$) and 32 (Clostridiaceae, $P = 0.005$) were increased in the asthmatic group (Figure 1A, Supplementary Table S6) whereas OTUs 5 (Clostridiales, $P = 0.046$) and 3 (*Lachnospira*, $P = 0.098$) were decreased in asthmatics. At 1 year of age, six differentially abundant OTUs were identified. Of note, three of these OTUs were classified

into the family Lachnospiraceae (one was statistically significant; OTU 40, $P = 0.032$; Figure 1B, Supplementary Table S6). Additionally, two other FLVR bacteria (*Veillonella* and *Rothia*) were increased in asthmatics at 1-year, though only *Rothia* was statistically significant ($P = 0.003$; Figure 1B, Supplementary Table S6).

Independent validation of 16S ribosomal RNA sequencing

In an effort to identify, more specifically, bacteria that could be used as biomarkers or probiotic treatments for asthma, we chose to validate these sequencing findings only for those OTUs classified down to the genus level (i.e. *C. neonatale*, *Lachnospira*, *Veillonella* and *Rothia*) using qPCR. 16S sequencing uses barcoded primers to amplify a hypervariable region of the 16S gene, whereas qPCR uses taxon-specific primers for amplification from metagenomic DNA. This makes qPCR an effective validation method for 16S sequencing results. Thus, informed by our findings from 16S sequence analysis (Figure 1), we designed and optimized genus-specific primers for the genera, *Lachnospira* and *Rothia*, and species-specific primers for the species, *C. neonatale*. We used previously published primers for *Veillonella* [7] (Supplementary Table S1, 3 months $n_{\text{asthmatic}} = 33$, $n_{\text{control}} = 24$; 1 year $n_{\text{asthmatic}} = 35$, $n_{\text{control}} = 28$). Subjects were included in this analysis based on sample availability and these subsets were determined to be representative of the larger cohort using a

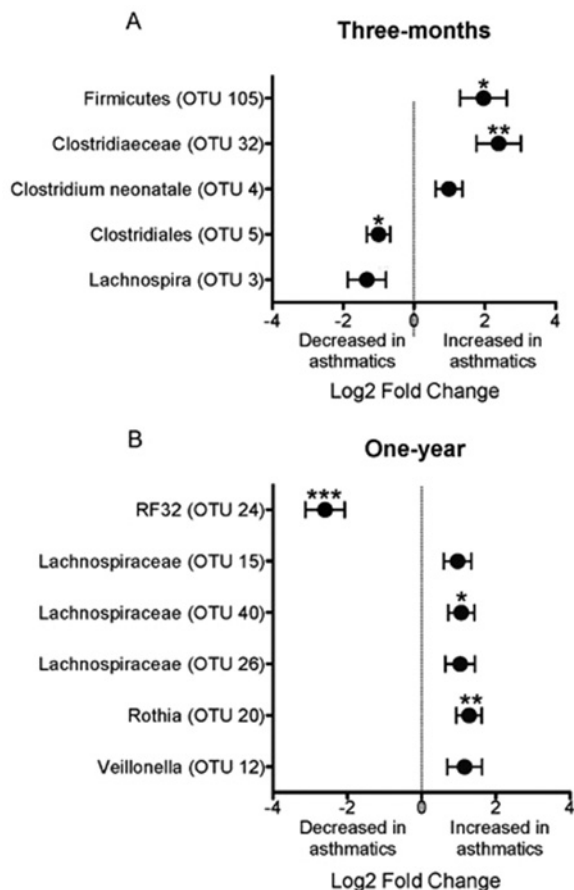


Figure 1 Differentially abundant OTUs identified by Deseq2 analysis at (A) 3 months and (B) 1 year

Each circle represents a specific OTU. An alpha threshold of 0.1 after Benjamini–Hochberg (for FDR) correction was used as a cutoff to identify these OTUs. Significant OTUs are specified as follows; $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ *** [3 months: Clostridiaceae OTU 32 $P = 0.005$; *C. neonatale* OTU 4 $P = 0.076$; Clostridiales OTU 5 $P = 0.035$; *Lachnospira* OTU 3 $P = 0.098$; Firmicutes OTU 105 $P = 0.035$; 1 year: RF32 OTU 24 $P = 3.64 \times 10^{-5}$; Lachnospiraceae OTU 15 $P = 0.078$, OTU 40 $P = 0.032$, OTU 26 $P = 0.078$; *Rothia* OTU 20 $P = 0.003$; *Veillonella* OTU 12 $P = 0.098$]. n asthmatics = 39, n controls = 37. Error bars represent standard error of the log₂ fold change.

logistic regression model (Supplementary Tables S2–S5). qPCR identified a significant reduction in the abundance of *Lachnospira* in the 3-month faecal microbiota but not the 1-year faecal microbiota of asthmatics compared with controls (Figure 2A, Mann–Whitney $P_{3\text{months}} = 0.008$). No significant differences in the abundance of *Veillonella* or *Rothia* were observed between asthmatics and controls at 3 months or 1 year (Supplementary Figure S3). Further, analysis by qPCR did not confirm a significantly higher abundance of *C. neonatale* in asthmatics at 3 months (Figure 2B). At 1 year however, qPCR did identify a significantly lower abundance of this taxon in asthmatics (Figure 2B, Mann–Whitney $P = 0.02$).

Interpreting these results as fold-changes relative to the control group further elucidates these apparent shifts in abundance. According to these qPCR findings, at 3 months asthmatic subjects were colonized with 1/5 less *Lachnospira* and 31 times more *C.*

neonatale. Although at 1 year, asthmatics were colonized with 16 times more *C. neonatale* and showed no difference in *Lachnospira* colonization. These opposing shifts in *Lachnospira* and *C. neonatale* lead us to hypothesize that a ratio calculation of *Lachnospira*/*C. neonatale* may be a quantifiable indicator of dysbiosis in asthmatic subjects.

Lachnospira/*C. neonatale* ratio to quantify dysbiosis

To assess if the relationship between these two bacteria is a quantifiable measure of dysbiosis related to preschool age asthma development, we calculated the L/C ratio for asthmatics and controls based on the RQ values from the qPCR analysis. At 3 months, the L/C ratio was significantly lower in asthmatics compared with controls (Figure 3A, Mann–Whitney $P = 0.008$). Calculating the ratio of *Lachnospira* to *C. neonatale* using the 16S rRNA read counts normalized to relative abundance confirmed this association (Mann–Whitney $P = 0.0001$). Interestingly, at 1 year a positive association was observed between the L/C ratio and the asthmatic phenotype (Figure 3B, Mann–Whitney $P = 0.049$), though the 16S rRNA read count ratio did not confirm this.

Notably, we did not identify any significant differences between asthmatics and controls after calculating ratios using the RQ values for *Veillonella* and *Rothia* in combination with *Lachnospira* and *C. neonatale* at 3 months (R/C, L/R, V/C, L/V, Supplementary Figure S1). At 1 year we did identify significant differences between asthmatics and controls for both the R/C and V/C ratios, suggesting that this decrease is mediated solely by the abundance of *C. neonatale*.

Further, the 3-month qPCR findings (specifically, the decrease in *Lachnospira* and the L/C ratio) are independent of antibiotic exposure, which is commonly associated with disturbances to the intestinal microbiota (Supplementary Figures S4 and S5). Sub-group analyses aimed at parsing out the specificity of these associations with atopic disorders in general did not identify significant differences between atopic and non-atopic asthmatics and the decreases in *Lachnospira* and the L/C ratio remained significant after excluding subjects diagnosed with AD at 3 months or 1 year or with parental history of asthma (Supplementary Figures S3, S4 and S6–S13). However, the decrease in *C. neonatale* and the increase in the L/C ratio at 1 year were not independent of these exposures. Thus in aggregate, these specificity analyses suggest that the diagnostic potential for these two particular bacterial taxa alone or as a ratio is greater if analysed in the first 3 months of life (Figures S3, S4 and S6–S13).

Quartile analysis of the *Lachnospira*/*C. neonatale* ratio

To assess this ratio at higher fidelity and to determine its potential as a microbe-based diagnostic technique, we analysed the L/C ratios at 3 months and 1 year as quartiles. Quartiles were determined based on the median and range of the qPCR RQ values and allowed for the categorization of these values into dichotomous variables ranging from the lowest L/C ratios (quartile 1) to the highest L/C ratios (quartile 4). ORs were calculated for each quartile; an OR above 1 is associated with higher odds of developing asthma, whereas an OR below 1 is associated with lower odds of developing asthma. At 3 months, the OR of being

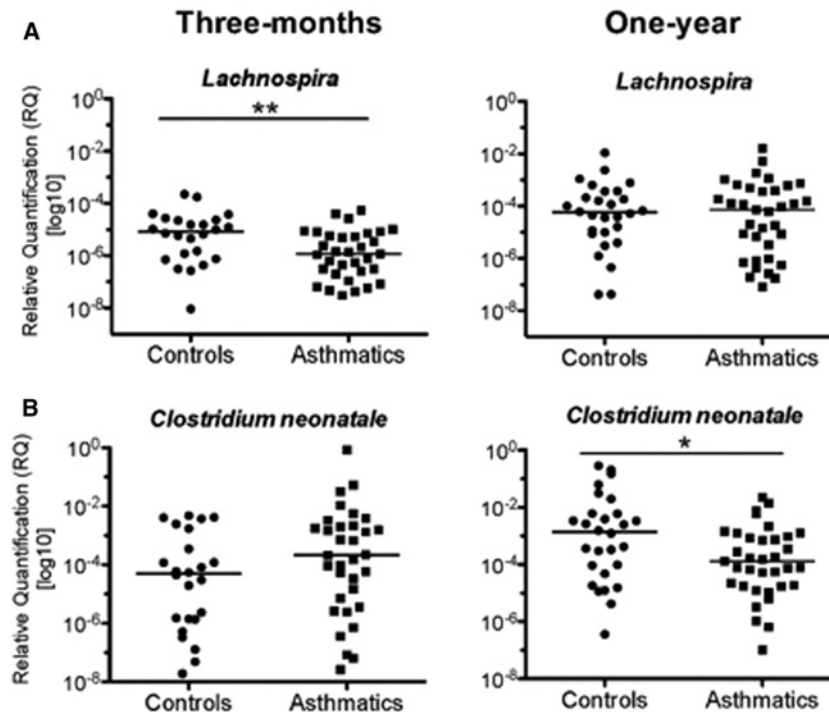


Figure 2 qPCR validation of 16S sequencing for *Lachnospira* and *C. neonatale* in the 3-month and 1-year faecal microbiota. (A) qPCR quantification of *Lachnospira* in the 3-month and 1-year gut microbiota. Mann-Whitney: 3 months $P=0.008$, 1 year (ns). (B) qPCR quantification of *C. neonatale* in the 3-month and 1-year gut microbiota. Mann-Whitney: 3 months (ns), 1 year $P=0.02$. Line represents the median; n_{3mo} Asthmatic = 33, n_{3mo} Control = 24, n_{1y} Asthmatic = 35, n_{1y} Control = 28. $P \leq 0.05^*$, $P \leq 0.01^{**}$.

classified into the asthmatic group decreases as the quartiles increase (as the ratio of L/C increases), with a plateau after quartile 3 (OR quartile 1 = 15, $P=0.004$, FDR adjusted $P=0.02$; OR quartile 2 = 0.96, ns (not significant); OR quartile 3 = 0.37, ns; OR quartile 4 = 0.44, ns), suggesting a protective effect against asthma development associated with increases in the L/C ratio at 3 months (Figure 3C, Supplementary Table S7). At 1 year there were no significant associations, reinforcing the importance of the first 100 days of life as the critical window in which microbial biomarkers for identifying subjects at high risk of asthma are most applicable (Figure 3C, Supplementary Table S7).

In addition to the significant associations between the L/C ratio and asthma diagnosis, quartile analysis yielded similar trends when *Lachnospira* and *C. neonatale* were analysed individually, but similar to the L/C ratio, these trends were only apparent at the 3-month time point (Supplementary Figure S14, Supplementary Table S7). Consequently, these results support quantification of microbial dysbiosis in the first 3 months of life by calculating the ratio of *Lachnospira* to *C. neonatale*, but the individual effects of these two bacterial taxa should also be taken into account.

DISCUSSION

Through our assessment of the intestinal microbiome among asthmatic and control children, we found evidence of bacterial dys-

biosis in the 3-month stool of children diagnosed with asthma by 4 years of age. Specifically, we found a reduction in the abundance of *Lachnospira*, and an increase in the species, *C. neonatale*, in the 3-month faecal microbiota of asthmatic children. These findings extend our previous work where we identified four bacterial genera (FLVR) that were less abundant in 3-month stool samples of children identified with atopy and wheezing at age 1 year [7]. Firstly, we show that a reduction in *Lachnospira* (one of the FLVR bacteria associated with atopic wheezing children) is a potential indicator of asthma diagnosed in preschool age children. Further, the present study supports the first 3 months of life as the early life 'critical window' in which the human immune system is most influenced by changes in gut microbiome composition.

Both *Lachnospira* and *C. neonatale* are intriguing bacteria with biologically compelling links to asthma and allergic disease. Although little is currently known about *C. neonatale*, recent research has implicated this species in neonatal necrotizing enterocolitis and proposes its classification into the *Clostridium* genus *sensu stricto* (Cluster I) [26]. Consistent with our findings, *Clostridium* Cluster I has been positively correlated with AD in humans [27], raising the possibility that this particular Cluster I species may play a role in other atopic disorders (such as asthma). In addition to our previous work identifying a reduction in *Lachnospira* in children at the highest risk of asthma development [7], *Clostridium* cluster XIVa (which includes *Lachnospira*) has been shown to promote colonic regulatory T-cell accumulation and lower levels of ovalbumin-specific IgE [28]. The individual

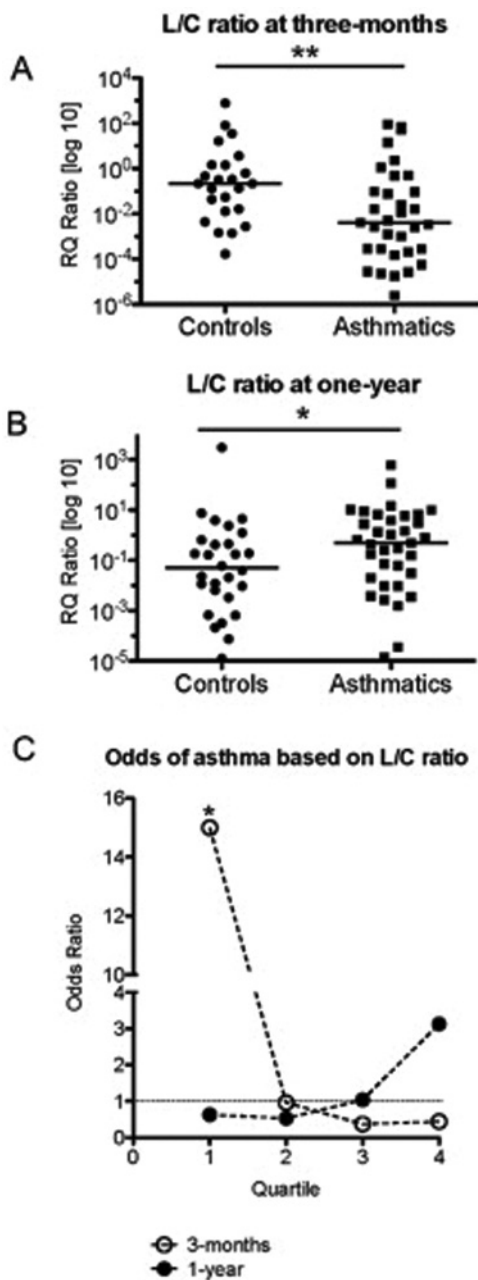


Figure 3 Ratio assessment and quartile analysis of *Lachnospira* and *C. neonatale*

Ratio of L/C RQ values at (A) 3 months and (B) 1 year. Line represents the median; n_{3mo} Asthmatic = 33, n_{3mo} Control = 24; Mann-Whitney $P = 0.008$; n_{1y} Asthmatic = 35, n_{1y} Controls = 28; Mann-Whitney $P = 0.048$. (C) Line graph representing the likelihood of asthma diagnosis based on quartile analysis of the L/C ratios at 3 months and 1 year (i.e. quartile 1 = low L/C ratio, quartile 4 = high L/C ratio). 3 months; quartile 1: OR = 15, $P = 0.004$, P adj. = 0.02, CI = 1.8–124.7; quartile 2: OR = 0.96, ns; quartile 3: OR = 0.37, ns; quartile 4: OR = 0.44, ns. 1 year; quartile 1: OR = 0.63, ns; quartile 2: OR = 0.53, ns; quartile 3: OR = 1.04, ns; quartile 4: OR = 3.13, ns. Points above the dotted line indicate increased odds of developing asthma; points below the dotted line indicate decreased odds of developing asthma. Stars indicate significant ORs; $P \leq 0.05^*$.

opposing shifts in the abundance of *Lachnospira* and *C. neonatale* in the first 3 months of life suggest that these specific gut bacterial taxa play a role in protecting (in the case of *Lachnospira*) or promoting (in the case of *C. neonatale*) the development of a preschool age asthmatic phenotype, in addition to their previously identified roles in other atopic disorders.

These findings are supported by analysis of the L/C ratio, which is significantly lower in asthmatics at 3 months of age. Associative quartile analysis of the L/C ratio with odds of asthma development further supports this association, with the odds of asthma development decreasing as the L/C ratio increases. This ratio was calculated as a quantifiable measure of dysbiosis based on two bacterial taxa; however, this does not negate the associations observed with the two bacteria individually (specifically the reduction in *Lachnospira* at 3 months). Quartile analysis of the L/C ratio and *Lachnospira* at 3 months identified children at a lower odds of developing asthma (L/C ratio: OR quartile 3 = 0.37, ns; OR quartile 4 = 0.44, ns) with the *Lachnospira* analysis identifying children at the lowest odds (OR quartile 4 = 0.12, $P = 0.002$, adj. $P = 0.008$). Only quartile analysis of the L/C ratio, however, identified children with the highest odds of developing preschool age asthma (quartile 1 OR = 15, $P = 0.004$, adj. $P = 0.02$), an important clinical finding with regard to early asthma diagnosis and potential prevention of this disease. For example, it could be possible to use the L/C ratio as a biomarker for the identification and prediction of subjects with increased potential to develop asthma later in life.

Collectively, these results expand on the current knowledge of the role of the intestinal microbiome in atopic disease, supporting the roles of specific gut bacteria in promoting or protecting against asthma development in children. However the aetiology of asthma is complex, as asthma and other atopic disorders are highly intertwined through the ‘atopic march’ of disease progression in early childhood. The qPCR results at 3 months are not influenced by parental history of asthma or AD in the first year of life and we found no significant differences between atopic and non-atopic asthmatics, as highlighted in the sub-group analyses (Figures S6–S13). However our study cohort was enriched for atopic children and the control subset chosen based on the absence of atopic disorders in the first 3 years of life, making it difficult to determine whether these particular bacteria are specific to asthma or also associated with other preschool age allergic diseases. Thus, it is possible that *Lachnospira* and *C. neonatale* are associated with other atopic disorders and it will be important for future studies to determine the diagnostic and probiotic potential of these taxa in atopic diseases in general.

Further, as identified in our previous work, the present study supports the first 100 days of life as the early life ‘critical window’ during which changes to the intestinal microbiome are most influential in promoting the development of IgE-mediated hypersensitivities in humans [7]. The 3-month findings also possess the greatest diagnostic potential as quartile analysis of the L/C ratio identified children at the highest risk of asthma development and *Lachnospira* analysis identified children at the lowest risk. However future studies should include repeated microbiome analyses beginning before 3 months and continuing up to 1 year of age to

more accurately define this early life critical window in humans. Lastly, the present study does not provide causative evidence for the role of these bacterial taxa in asthma development, though we did previously demonstrate that *Lachnospira* (along with the three other FLVR bacteria) ameliorated lung inflammation in an OVA-challenged mouse model [7]. Additional translational studies combining human and animal research are necessary to mechanistically define how these bacterial taxa protect against or promote hypersensitivity diseases like asthma.

In conclusion, the present study highlights two Clostridial species with potentially contrasting roles in the development of preschool asthma—*Lachnospira* and *C. neonatale*. Assessment of these bacteria as a ratio (L/C) represents a novel quantification method for measuring taxon-specific gut dysbiosis. Additionally, the present study emphasizes the importance of the first 100 days of life as the critical window during which transient gut microbial dysbiosis is associated with immune dysregulation and asthma later in life. Moving forward, this work will inform the development of biomarkers to predict risk of asthma and the establishment of rationally designed probiotic regimens to protect children from asthma.

CLINICAL PERSPECTIVES

- The intestinal microbiota has been implicated as a therapeutic target for atopic disease, but little is known about the role of the gut microbiota in children diagnosed with asthma.
- Here we show that opposing shifts in the relative abundance of specific bacterial taxa, *Lachnospira* and *C. neonatale*, are associated with asthma diagnosed by 4 years of age.
- Assessment of these bacterial shifts as a ratio (L/C) represents a novel method of quantifying taxa-specific intestinal dysbiosis and could be used in the identification of subjects at high risk of developing preschool age asthma.

AUTHOR CONTRIBUTION

All authors contributed extensively to this work. Leah Stiemsma, Stuart Turvey and Brett Finlay designed the study. Diana Lefebvre, Padmaja Subbarao, Piush Mandhane, Allan Becker, Malcolm Sears, Meghan Azad and CHILD Study Investigators made CHILD Study samples possible and accessible. Meghan Azad curated all breast-feeding data. Marie-Claire Arrieta and Pedro Dimitriu optimized sequencing strategy. Leah Stiemsma curated all metadata, classified subjects into asthmatic and control groups, and performed all statistical analyses. Leah Stiemsma and Lisa Thorson prepared all stool samples for sequencing. Leah Stiemsma and Jasmine Cheng designed qPCR strategy and performed qPCR analysis. Leah Stiemsma analysed qPCR and sequencing results. Leah Stiemsma and Stuart Turvey wrote the manuscript. All authors edited and approved the manuscript.

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DECLARATIONS OF INTEREST

L.T.S., M.-C.A., B.B.F. and S.E.T. filed a patent (PCT/CA2016/000,065) entitled "Bacterial Composition and Methods of Use Thereof" on March 11, 2016. There are no other competing interests in relation to the work described in this manuscript.

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