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

Leah T. Stiemsma, Marie-Claire Arrieta, Pedro A. Dimitriu, Jasmine Cheng, Lisa Thorson, Diana L. Lefebvre, Meghan B. Azad, Padmaja Subbarao, Piush Mandhane, Allan Becker, Malcolm R. Sears, Tobias R. Kollmann, the Canadian Healthy Infant Longitudinal Development (CHILD) Study Investigators, William W. Mohn, B. Brett Finlay, Stuart E. Turvey

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 one-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 analyze 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), while the abundance of the species, *Clostridium neonatale* (C), was increased (p = 0.07) in asthmatics. Quartile analysis revealed a negative association between the ratio of these two bacteria (L/C) and asthma risk at 3-months (quartile 1: Odds ratio (OR) = 15, p = 0.02, CI = 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.

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

Leah T. Stiemsma[1, 2], Marie-Claire Arrieta[1,3], Pedro A. Dimitriu[1], Jasmine Cheng[2], Lisa Thorson[3], Diana L. Lefebvre[4], Meghan B. Azad[5], Padmaja Subbarao[6, 7], Piush Mandhane[8, 9], Allan Becker[5], Malcolm R. Sears[4], Tobias R. Kollmann[2, 10], the Canadian Healthy Infant Longitudinal Development (CHILD) Study Investigators[11], William W. Mohn[1], B. Brett Finlay[1, 3, 12]*, Stuart E. Turvey[2, 10]*

[1]Department of Microbiology & Immunology, University of British Columbia, Vancouver, BC. [2]Child & Family Research Institute and BC Children's Hospital, Vancouver, BC. [3]Michael Smith Laboratories, University of British Columbia, Vancouver, BC. [4]Department of Medicine, McMaster University, Hamilton, ON. [5]Department of Pediatrics and Child Health, University of Manitoba, Winnipeg, MB. [6]Department of Pediatrics, University of Toronto, Toronto, ON. [7]Hospital for Sick Children, Toronto, ON. [8]Department of Pediatrics, University of Alberta, Edmonton, AB. [9]School of Public Health, University of Alberta, Edmonton, AB. [10]Department of Pediatrics, University of British Columbia, Vancouver, BC. [11]CHILD Study investigators are listed in the Online Data Supplement. [12]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC.

Corresponding Authors:
Stuart Turvey MBBS DPhil FRCPC
Director of Clinical Research; Child & Family Research Institute
Aubrey J. Tingle Professor of Pediatric Immunology
Professor, The University of British Columbia
950 W 28th Ave, Vancouver, BC, V5Z 4H4
Canada
Email: sturvey@cfri.ca

Brett Finlay OC, OBC, FRSC, FCAHS
UBC Peter Wall Distinguished Professor
CIFAR Senior Fellow
Michael Smith Laboratories
#301-2185 East Mall, University of British Columbia
Vancouver, BC, V6T 1Z4
Canada
Email: bfinlay@mail.ubc.ca

*These authors made an equal contribution.

SHORT TITLE: Opposing shifts in *Lachnospira* and *Clostridium sp.* are associated with asthma

KEYWORDS: gut microbiota, atopic disease, dysbiosis, hygiene hypothesis, microflora hypothesis

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 one-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 analyze 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), while the abundance of the species, *Clostridium neonatale* (C), was increased (p = 0.07) in asthmatics. Quartile analysis revealed a negative association between the ratio of these two bacteria (L/C) and asthma risk at 3-months (quartile 1: Odds ratio (OR) = 15, p = 0.02, CI = 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.

ABBREVIATIONS: CHILD Study (Canadian Healthy Infant Longitudinal Development Study), ISAAC (International Study of Asthma and Allergies in Childhood), qPCR (quantitative polymerase chain reaction), FDR (false discovery rate), OTU (operational taxonomic unit), L/C (*Lachnospira/C. neonatale*), OR (odds ratio)

SUMMARY STATEMENT: Opposing shifts in the abundances of *Lachnospira* and *C. neonatale* in the 3 month intestinal microbiota are associated with asthma in preschool age children and are potential early life indicators of asthma risk.

INTRODUCTION

Asthma is a multifactorial disease driven by both genetic and environmental factors. While 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 generation (2). 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 (3). Instead this dysbiosis skews the immune system toward immune-mediated and hypersensitivity disorders (4, 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 one-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 four 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 four 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 three months of life and the odds of developing asthma by four years of age. This ratio, in combination with the individual shifts in these two taxa in the first 100 days of life, may have potential important clinical implications with regard to asthma diagnosis and prevention.

METHODS

CHILD study design and ethics approval: The Canadian Healthy Infant Longitudinal Development (CHILD) Study is a longitudinal, general population birth cohort composed of 3,624 families recruited at four sites across Canada (Vancouver, Edmonton, Manitoba, Toronto). The study follows infants from pregnancy to five 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-weeks 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, 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: This study is based on a nested case-control design and comprised of subjects enrolled in the CHILD study that were analyzed 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 four years of age or was prescribed inhaled asthma medications (inhaled corticosteroids or bronchodilators) from three – four 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 one- and three-years of age) and negative for wheezing (based on questionnaire analysis repeated 6 times from birth – four 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 atopic dermatitis (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-

149 year clinical assessment or a non-CHILD clinician as reported in one-year CHILD health
150 questionnaire). 'No' = no diagnosis.
151
152 *Feeding methods:* Continuous covariate defined by the duration (in months) a child was breast
153 fed.
154
155 *Parental history of asthma:* Defined as neither parent having asthma or at least one parent having
156 asthma. Reference level is neither parent.
157
158 *Delivery mode:* Reference is cesarean section birth.
159
160 *Sex:* Reference is female.
161
162 *Microbial community analysis:* Full details regarding our 16S rDNA extraction, PCR
163 amplification, and bioinformatics have been previously described (7). Briefly, DNA was
164 extracted from 3-month and 1-year stool samples using Mo-bio dry bead tubes (Mo Bio
165 Laboratories), the Fastprep homogenizer (FastPrep Instrument, MP Biochemicals) or the
166 Disruptor Genie (Scientific Industries, Inc.) and the Qiagen DNA stool mini kit.
167
168 DNA samples were amplified by PCR in triplicate using barcoded primer pairs spanning the V3
169 region of the 16S gene (7, 14). V3 PCR amplicons were sequenced using Hi-Seq 2000
170 bidirectional Illumina sequencing (Macrogen Inc.). Sequences were quality filtered and denoised
171 using Mothur (15) and clustered into operational taxonomic units (OTUs) using CrunchClust

172 (16). Clusters were classified against the Greengenes Database (17) according to 97% similarity
173 (Levenshtein distance = 5). OTUs with a frequency less than five among all samples were
174 excluded.
175
176 *qPCR primer design and validation:* Sequences for the 16S rRNA genes of the bacterial genera
177 and species of interest and of closely related bacteria were aligned by CLUSTAL-W using
178 MEGA6 alignment explorer and inspected for conserved and variable regions. Based on this
179 analysis, we designed genus-specific primer candidates for *Lachnospira* and *Rothia* and species-
180 specific primer candidates for *C. neonatale*. Primer candidates were assessed for specificity
181 against all bacterial sequences using Primer-Blast. The primer melting temperature, secondary
182 structure and dimer formation, and G+C content were analyzed using OligoAnalyzer3.1
183 (Integrated DNA Technologies). Primer pairs meeting all these requirements were validated
184 using the standard curve method in metagenomic DNA extracted from human fecal samples
185 (**Table S1**).
186
187 *Quantitative PCR conditions:* Each 10μL reaction contained 5μL of IQ SYBR green supermix
188 (Bio-Rad), 0.1μL of each forward and reverse primer, 0.8μL of nuclease-free water, and 4μLof
189 fecal DNA extract. All reactions were carried out in the ViiA 7 Real-Time PCR System (Life
190 Technologies Inc.) under the following conditions: an initial step at 95 ^{o}C (5 min), 40 cycles of
191 15s at 94 ^{o}C, 30s at the specific annealing temperature for each primer set (**Table S1)**, 30s at 72
192 ^{o}C (*C. neonatale, Veillonella* (7), and Bacteria (18)) or 20s at 72 ^{o}C (*Rothia*, and *Lachnospira*),
193 and a final cycle of 95 ^{o}C at 15s, 60 ^{o}C at 1 min, 95 ^{o}C at 15s, and 60 ^{o}C at 15s. All samples were
194 run in triplicate and normalized according to the ΔC_T method using total 16S rDNA (Bacteria

(18), **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 was 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. Ln(OR) above 0 implies an increased likelihood that a child would develop asthma, while 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 this study were representative of the entire cohort (**Tables S4-S7**).

16S sequence analysis: The microbial diversity of the fecal microbiota (based on the Shannon alpha diversity index) of asthmatics and controls was analyzed in Phyloseq (20). Deseq2 (21) was used to calculate the multi-inference adjusted p-values (based on false discovery rate, FDR) and log2 fold changes associated with differentially abundant OTUs between asthmatics and controls. Principal components analysis (PCA) was conducted using MetaboAnalyst (22, 23).

This 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 qPCR analyses were carried out using GraphPad Prism version 5c.

Calculation of bacterial ratios and quartile analysis: All ratios (**Fig. 3 & S13**) 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 L/C ratio 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: This study comprised 286 subjects enrolled in the CHILD study and analyzed in our previous report (7) who had reached three years of age at the time this study began. Of these 286 subjects, 39 met our criteria for asthma based on physician diagnosis or

241 having been prescribed medications used to treat asthma by four years of age (asthmatic group).
242 For comparison, we identified 37 control subjects who had no evidence of asthma or allergic
243 disease. These control subjects were negative for asthma and also negative for atopy and
244 wheezing from birth to three years of age. Asthmatic and control subjects were matched for
245 gender, birth mode (vaginal vs. caesarean section), feeding practices (breast fed vs. formula fed),
246 and antibiotic exposure **(Table 1)**. In line with previous studies, children diagnosed with atopic
247 dermatitis (AD) at 1-year of age or those with parental history of asthma were more likely to
248 develop preschool age asthma (ln(OR) 1-year AD: 1.68, CI = 0.06 – 3.13, p = 0.04; ln(OR)
249 parental history = 1.51, CI = 0.43 – 2.6, p = 0.006, **Table 1**) (25).

250

251 *Microbial community analysis by 16S ribosomal RNA gene amplicon sequencing suggests a role*
252 *for Lachnospira and C. neonatale:* The global gut microbial community composition in stool
253 samples taken at 3-months or 1-year of age did not differ between asthmatics and controls (as
254 shown by principal components analysis and analysis of microbial diversity at 3-months and 1-
255 year **(Fig. S1).** Beyond the analysis of global microbial community composition, we used
256 Deseq2 with Benjamini-Hochberg adjustment (for FDR at an alpha threshold of 0.1) to identify
257 differentially abundant operational taxonomic units (OTUs) between asthmatics and controls at
258 3-months or 1-year; with statistical significance defined as $P \leq 0.05$. At 3 months of age, five
259 differentially abundant OTUs were identified (**Fig. 1A, Table S2**). Of note, OTUs 4 (*C.*
260 *neonatale*, p = 0.076) and 32 (Clostridiaceae, p = 0.005) were increased in the asthmatic group
261 (**Fig. 1A, Table S2**) while OTUs 5 (Clostridiales, p = 0.046) and 3 *(Lachnospira*, p = 0.098)
262 were decreased in asthmatics. At 1 year of age, six differentially abundant OTUs were identified.
263 Of note, three of these OTUs were classified into the family Lachnospiraceae (one was

statistically significant; OTU 40, p = 0.032; **Fig. 1B, Table S2**). Additionally, two other FLVR bacteria (*Veillonella* and *Rothia*) were increased in asthmatics at one-year, though only *Rothia* was statistically significant (p = 0.003; **Fig. 1B, Table S2**).

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 quantitative PCR (qPCR). 16S sequencing uses barcoded primers to amplify a hypervariable region of the 16S gene, while 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 (**Fig. 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)* (**Table S1**, three-months $n_{asthmatic} = 33$, $n_{control} = 24$; one-year $n_{asthmatic} = 35$, $n_{control} = 28$). Subjects were included in this analysis based on sample availability and these subsets were determined to be representative of the larger cohort using a logistic regression model (**Tables S4-S7**). qPCR identified a significant reduction in the abundance of *Lachnospira* in the 3-month fecal microbiota but not the 1-year fecal microbiota of asthmatics compared to controls (**Fig. 2A,** Mann-Whitney $p_{3months} = 0.008$). No significant differences in the abundance of *Veillonella* or *Rothia* were observed between asthmatics and controls at 3-months or 1-year (**Fig. S2**). Further, analysis by qPCR did not confirm a significantly higher abundance of *C. neonatale* in asthmatics at 3-months (**Fig. 2B**). At 1-year

however, qPCR did identify a significantly lower abundance of this taxon in asthmatics (**Fig. 2B**, Mann-Whitney p = 0.02).

Interpreting these results as fold-changes relative to the asthmatic 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*. While 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 ratio of *Lachnospira/C. neonatale* (L/C) for asthmatics and controls based on the relative quantification values from the qPCR analysis. At 3-months, the L/C ratio was significantly lower in asthmatics compared to controls (**Fig. 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 (**Fig. 3B**, Mann-Whitney p = 0.049), though the 16S rRNA read count ratio did not confirm this.

Notably, we did not identify any significant differenes between asthamtics 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, **Fig. S13**). At 1-year we did identify significant differences between asthmatics and controls for both the R/C and V/C, 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 (**Fig. S5 & S6**). 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 (**Figs. S3, S4, & S7 – S12**). 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 analyzed in the first 3-months of life (**Figs. S3, S4, & S7 – S12**).

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 analyzed 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). Odds ratios (OR) were calculated for each quartile; an odds ratio above 1 is associated with higher odds of developing asthma, while an odds ratio below 1 is associated with lower odds of developing asthma. At 3-months, the odds ratio of being 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; 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 three-months (**Fig. 3C, Table S3)**. 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 (**Fig. 3C**, **Table S3**).

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 analyzed individually, but similar to the L/C ratio, these trends were only apparent at the 3 month time point (**Fig. S3, Table S3**). 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 dysbiosis 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 fecal 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 one 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, this 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 atopic dermatitis 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 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 etiology 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 atopic dermatitis 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 (**Figs. S3 – S12**). However our study cohort was enriched for atopic children and the control

399 subset chosen based on the absence of atopic disorders in the first three years of life, making it
400 difficult to determine whether these particular bacteria are specific to asthma or also associated
401 with other preschool age allergic diseases. Thus, it is possible that *Lachnospira* and *C. neonatale*
402 are associated with other atopic disorders and it will be important for future studies to to
403 determine the diagnostic and probiotic potential of these taxa in atopic diseases in general.

404 Further, as identified in our previous work, this study supports the first 100 days of life as the
405 early life 'critical window' during which changes to the intestinal microbiome are most
406 influential in promoting the development of IgE-mediated hypersensitivities in humans (7). The
407 3-month findings also possess the greatest diagnostic potential as quartile analysis of the L/C
408 ratio identified children at the highest risk of asthma development and *Lachnospira* analysis
409 identified children at the lowest risk. However future studies should include repeated
410 microbiome analyses beginning before 3 months and continuing up to 1 year of age to more
411 accurately define this early life critical window in humans. Lastly, this study does not provide
412 causative evidence for the role of these bacterial taxa in asthma development, though we did
413 previously demonstrate that *Lachnospira* (along with the three other FLVR bacteria) ameliorated
414 lung inflammation in an OVA-challenged mouse model (7). Additional translational studies
415 combining human and animal research are necessary to mechanistically define how these
416 bacterial taxa protect against or promote hypersensitivity diseases like asthma.

417

418 In conclusion, this study highlights two Clostridial species with potentially contrasting roles in
419 the development of preschool asthma—*Lachnospira* and *C. neonatale*. Assessment of these
420 bacteria as a ratio (L/C) represents a novel quantification method for measuring taxon-specific
421 gut dysbiosis. Additionally, this 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 four 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.

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DECLARATIONS OF INTERST: LTS, MCA, BBF, and SET filed a provisional patent 62/132,042, entitled “Intestinal bacterial composition and methods to detect and prevent

asthma," in the United States on 3 December 2014. There are no other competing interests in relation to the work described in this manuscript.

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AUTHOR CONTRIBUTIONS: All authors contributed extensively to this work. LTS, SET, and BBF designed the study. DLL, PS, PM, AB, MRS, MBA and CHILD Study Investigators made CHILD study samples possible and accessible. MBA curated all breast-feeding data. MCA and PD optimized sequencing strategy. LTS curated all metadata, classified subjects into asthmatic and control groups, and performed all statistical analyses. LTS and LT prepared all stool samples for sequencing. LTS and JC designed qPCR strategy and performed qPCR analysis. LTS analyzed qPCR and sequencing results. LTS and SET wrote the manuscript. All authors edited and approved the manuscript.

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TABLES:

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				

Abbreviations: OR = odds ratio, AD = atopic dermatitis, CI = confidence interval.

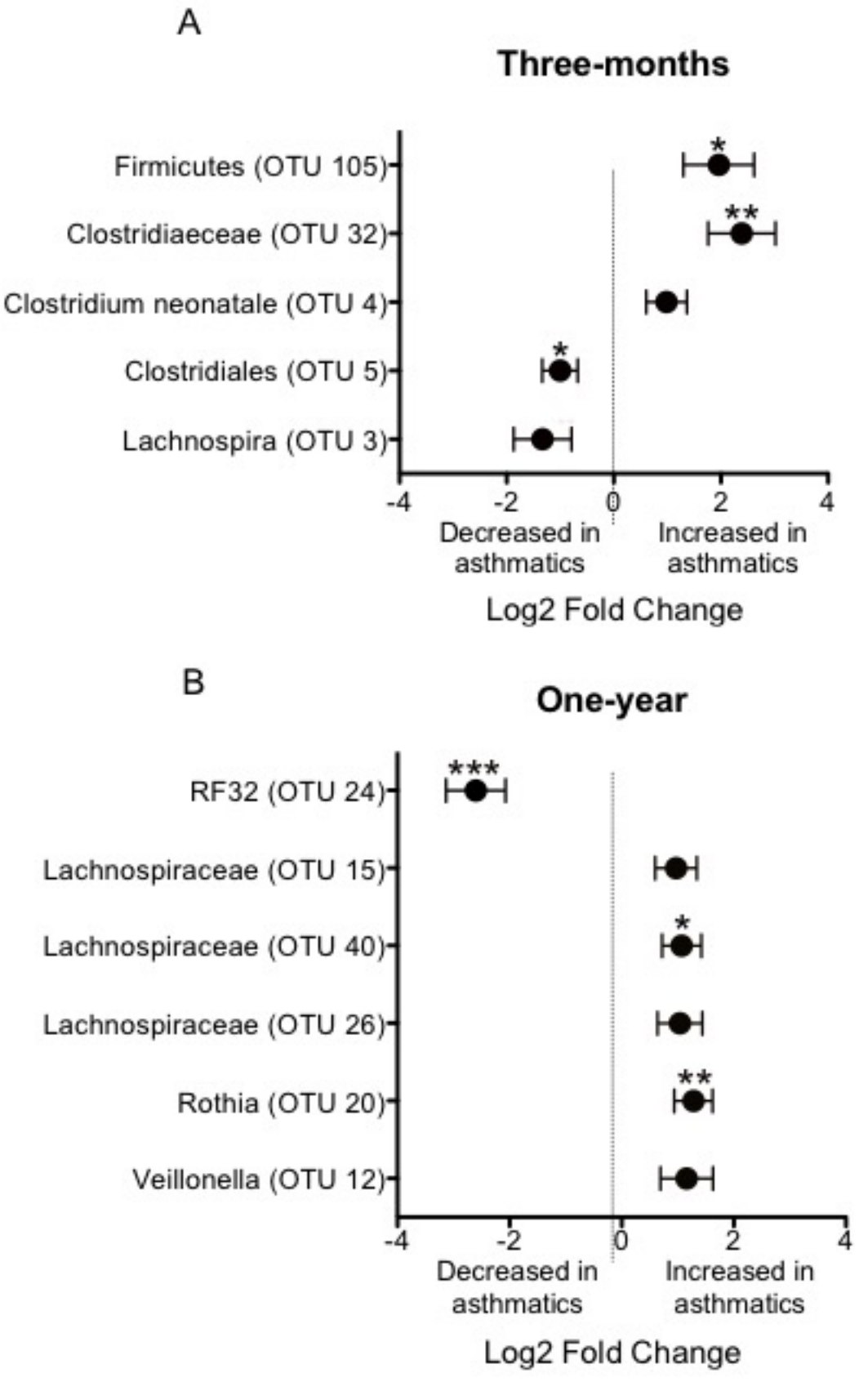
FIGURES:

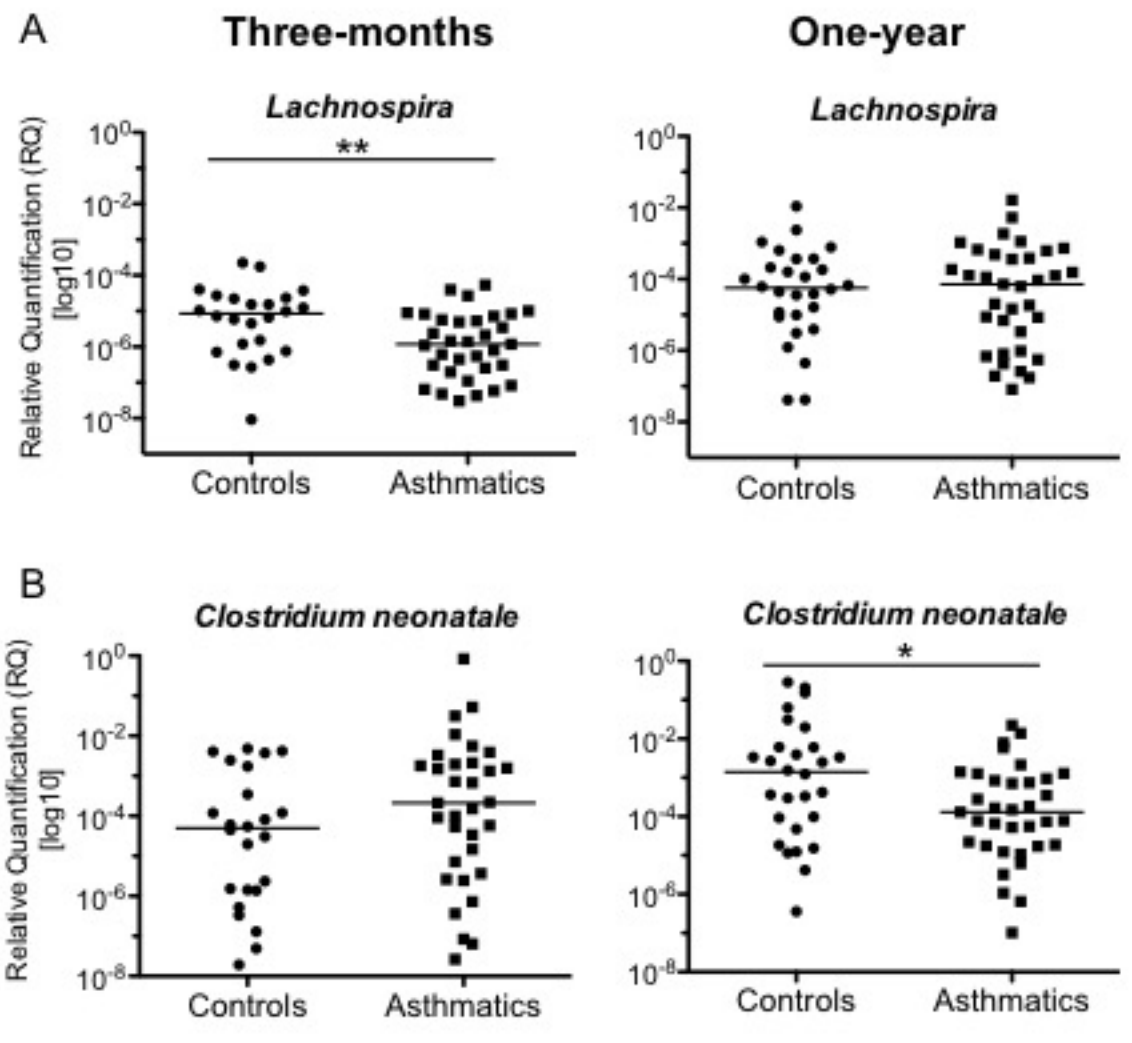
Figure 1: Differentially abundant OTUs identified by Deseq2 analysis at A) 3-months and B) 1-year. Each circle represents a specific OTU. An alpha threshold of 0.1 after Benjamini-Hochberg (for FDR) correction was used as a cutoff to identify these OTUs. Significant OTUs are specified as follows; p< 0.05 *, p<0.01 **, p<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; One-year: RF32 OTU 24 p = $3.64e^{-05}$; 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 numbers; n asthmatics = 39, n controls = 37. Error bars represent standard error of the log2 fold change.

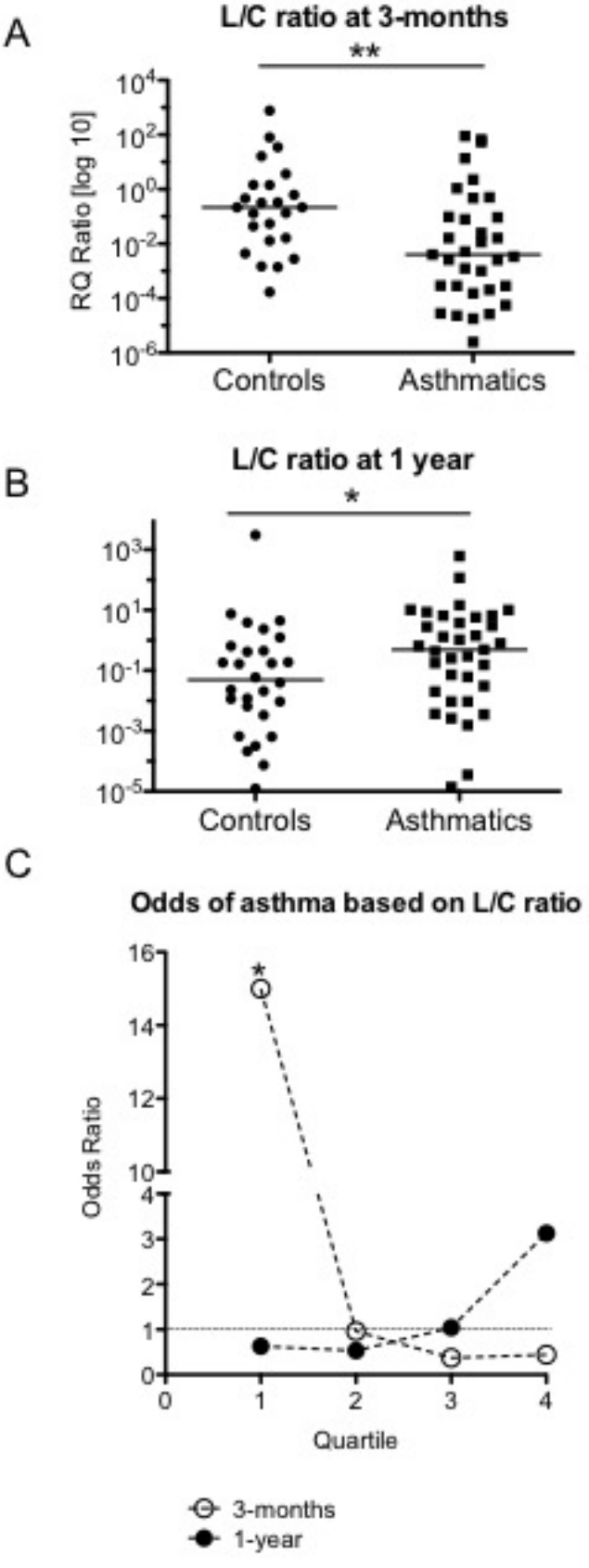
Figure 2: qPCR validation of 16S sequencing for *Lachnospira* and *C. neonatale* in the 3-month and 1-year fecal microbiota. A) qPCR quantification of *Lachnospira* in the 3-month and 1-year gut microbiota. Mann Whitney: 3-months p = 0.008, 1-year (ns). **B)** qPCR quantification of *C. neonatale* in 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. Star representation; p< 0.05*, p< 0.01**.

Figure 3: Ratio assessment and quartile analysis of *Lachnospira* and *C. neonatale*. Ratio of *Lachnospira/C. neonatale* (L/C) relative quantification (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; $p < 0.05$*.


A
Three-months
Firmicutes (OTU 105)
Clostridiaeceae (OTU 32)
Clostridium neonatale (OTU 4)
Clostridiales (OTU 5)
Lachnospira (OTU 3)
*
**
*
-4
-2
0
2
4
Decreased in asthmatics
Increased in asthmatics
Log2 Fold Change
B
One-year
RF32 (OTU 24)
Lachnospiraceae (OTU 15)
Lachnospiraceae (OTU 40)
Lachnospiraceae (OTU 26)
Rothia (OTU 20)
Veillonella (OTU 12)
***
*
**
-4
-2
0
2
4
Decreased in asthmatics
Increased in asthmatics
Log2 Fold Change


A
Three-months
One-year
Lachnospira
Lachnospira
**
Relative Quantification (RQ) [log10]
10^{0}
10^{-2}
10^{-4}
10^{-6}
10^{-8}
Controls
Asthmatics
B
Clostridium neonatale
Clostridium neonatale
*


A
L/C ratio at 3-months
**
RQ Ratio [log 10]
Controls
Asthmatics
B
L/C ratio at 1 year
*
Controls
Asthmatics
C
Odds of asthma based on L/C ratio
Odds Ratio
Quartile
3-months
1-year