Microbes and Infection 18 (2016) 543–549

www.elsevier.com/locate/micinf

Original article

High fecal IgA is associated with reduced *Clostridium difficile* colonization in infants

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Received 11 January 2016; accepted 4 May 2016
Available online 24 May 2016

Abstract

Colonization of infants with *Clostridium difficile* is on the rise. Although better tolerated by infants than adults, it is a risk factor for future allergic disease. The present study describes associations between infant fecal immunoglobulin A (IgA) and colonization with *C. difficile* in 47 infants enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) study.

C. difficile colonization was observed in over half (53%) of the infants. Median IgA was lower in infants colonized with *C. difficile* (10.9 μg versus 25.5 μg per g protein; $p = 0.18$). A smaller proportion of infants with IgA in the highest tertile were colonized with *C. difficile* compared to the other tertiles (31.3% versus 64.5%, $p = 0.03$). In unadjusted analysis, odds of colonization with *C. difficile* was reduced by 75% (OR 0.25 95% CI 0.07, 0.91 $p = 0.04$) among infants with IgA in the highest tertile compared to those in the other tertiles. Following adjustment for parity, birth mode and breastfeeding, this association was even stronger (aOR 0.17, 95% CI 0.03, 0.94, $p = 0.04$). Our study provides evidence that high fecal IgA, independent of breastfeeding, is associated with reduced likelihood of *C. difficile* colonization in infancy.

Keywords: Clostridium difficile; Immunoglobulin A; Infant

1. Introduction

Alongside its global rise as a cause of a debilitating infection [1], the enteric pathogen, *Clostridium difficile*, is being detected at an earlier age and in a greater number of infants than observed in the 1980s [2]. Up to 75% of healthy infants are colonized by *C. difficile* and, in some communities, 26% of them with toxic-producing strains [3]. In contrast to older children and adults who can develop severe diarrhea and colitis, carriage of *C. difficile* is well tolerated by infants [4]; they manifest no obvious symptoms from the toxins released by this anaerobe. The organism can be acquired in infancy from environmental contamination in the nursery or home environment [3]. Between 12 and 24 months of age, *C. difficile* abundance diminishes in gut microbiota. Yet, studies have

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[1] Canadian Healthy Infant Longitudinal Development Study (investigators listed in acknowledgements).

http://dx.doi.org/10.1016/j.micinf.2016.05.001
1286-4579/

shown that infants colonized with *C. difficile* are at increased risk for atopic outcomes later in childhood, including allergic sensitization, atopic dermatitis, recurrent wheeze and asthma [5–7]. Still other research suggests that associations with atopic disease are not due to *C. difficile* per se, but rather its presence may be a marker for lower colonization resistance. Changes in the ecosystem of gut microbiota in infants, such as higher abundance with genus *Ruminococcus* and *Klebsiella*, and lower abundance of *Bifidobacterium*, have been observed in the presence of *C. difficile* [8]. In adults, these changes are brought on with antibiotic use [9]; among infants, prior exposure to antibiotics is also risk factor for *C. difficile*, as is reduced exposure to human milk or lack of food diversity [3,10].

Although infants are better able to tolerate *C. difficile*, not all infants are colonized with this microbe; it is detected in only one third of infants under the age of 6 months [3]. What factors prevent colonization? Immunoglobulin A (IgA), in its secretory form (sIgA), is important in immune exclusion and the development of oral tolerance [11,12]. During the first weeks of life an infant's immune system is immature and its ability to produce IgA is limited [13]. Evidence from animal and human studies has demonstrated interactions between IgA and commensal gut bacteria whereby microbial exposure stimulates IgA production and in turn, IgA regulates the composition and activity of the microbiota (Fig. 1) [14–16]. Animal studies have shown that an absence of IgA in the intestine alters gut microbial composition and intestinal colonization of germ-free mice induces IgA responses [17,18]. Kukkonen et al. demonstrated a trend towards higher fecal IgA concentration following pre and probiotic treatment (*Lactobacillus* and *Bifidobacterium* species) of infants; antibiotic treatment was found to abolish the increase in fecal IgA in infants at 6 months following the administration of probiotics [19]. In a study on 64 Swedish infants, Sjögren et al. reported higher salivary IgA concentrations at 6 and 12 months following colonization with species of bifidobacteria [20]. In the same study, colonization at one month with *C. difficile* and lactobacilli was associated with increased IgA levels in children at age 1 and 5 years.

We previously reported on pre and postnatal predictors of fecal IgA levels in infants at 3–4 months of age [21]. To extend this work by better understanding the interactions between *C. difficile* and host immunity, we examined the association between infant fecal IgA concentration at this age and fecal colonization by *C. difficile*, independent of breastfeeding as a main source of IgA.

2. Materials and methods

2.1. Study design

A sub-sample of 47 infants (36–46 weeks gestation) from the Vancouver and Winnipeg sites of the Canadian Healthy Infant Longitudinal Development national population-based birth cohort (www.canadianchildstudy.ca) were included in the study for whom fecal samples were available for analysis [22]. Mothers of these infants were enrolled during pregnancy between September 2008 and January 2009. Stool samples were collected at mean age of 3.9 months (range 2.9–5.3) using a standard protocol as part of a scheduled home visit. Samples were refrigerated in the home immediately following collection and during transport and then stored at −80 °C for later use. At this time mothers were asked to report on breastfeeding status using a standardized questionnaire and breastfeeding was categorized as any breastfeeding (yes/no) and by degree of breastfeeding exposure (none, partially breastfed, exclusively breastfed). Information on other covariates were obtained from hospital records (infant sex, mode of delivery, birth weight, gestational age and maternal antibiotics

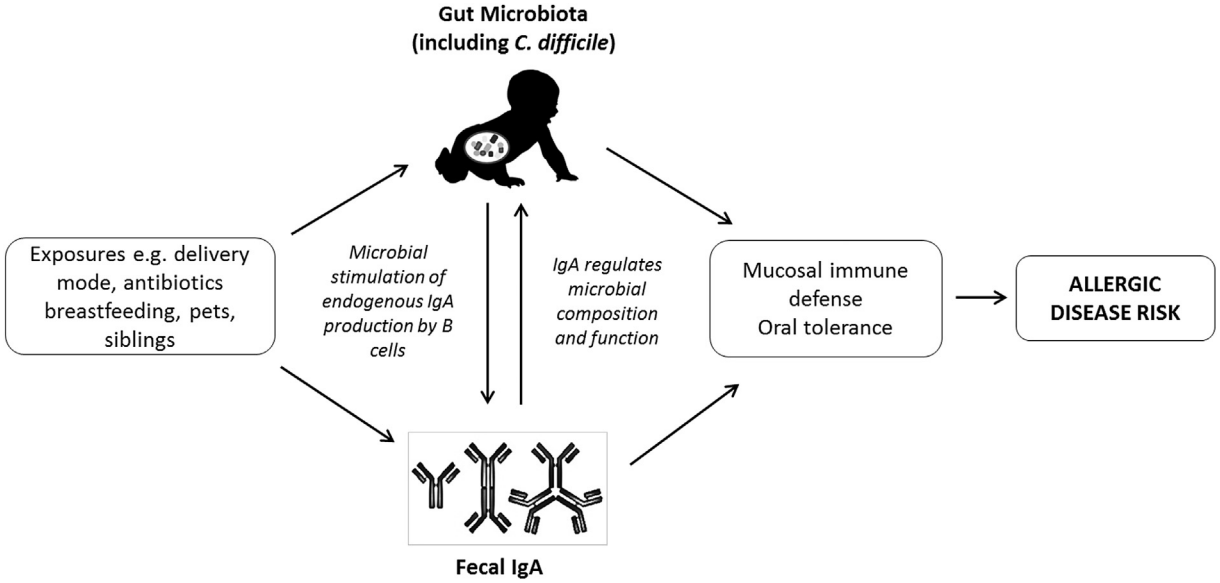

Fig. 1. Schematic representation of the potential inter-relationship between breastfeeding, gut microbiota, fecal IgA and allergic disease.

during birth) or through standardized questionnaires completed by mothers (maternal age, current maternal allergy or asthma, smoking during pregnancy, older maternal siblings as a proxy for parity, furry household pets, infant antibiotic use before 3 months). Maternal weight status (Body Mass Index weight kg/height meters^2) was calculated from height and weight measured at the 1 year post-partum clinic visit. Written informed consent was obtained from parents at enrollment.

2.2. Analysis of fecal samples

Fecal IgA from stool samples was quantified in duplicate using enzyme-linked immunosorbant assay (Bethyl Laboratories Human IgA kit, TX, USA) according to the manufacturer's instructions and expressed as average μg IgA per g total protein (total protein determined by standard colorimetric BCA protein assay). The manufacture's Accessory Kit supplied all buffers, plates and 3,3′,5,5′-Tetramethylbenzidine (TMB) and the color was allowed to develop for 10 min and then stopped with 50ul of 0.2 M sulfuric acid (Fisher Scientific, AB, CA). Absorbance was read at 450 nm using a scanning spectrophometer (Molecular Devices, CA USA). Samples with a CV greater than 10% were repeated.

We followed the method of Penders et al. for quantitative polymerase chain reaction for targeted analysis of *C. difficile* [23]. Oligonucleotides were manufactured by IDT (Integrated DNA Technologies Inc, Coralville, IA, USA). All reactions were performed on the MiniOpticonTM Real-Time PCR System (Bio-Rad, Hercules, CA, USA).

2.3. Statistical analysis

Spearman correlation was used to examine the association between fecal IgA and age of fecal collection, gestational age and birth weight. Univariate non-parametric statistics (Mann–Whitney U test, Kruskal Wallis) were used to describe associations between fecal IgA (as a continuous variable) and *C. difficile* colonization, as well as associations between IgA and other covariates. To assess non linear relationships, the same associations were tested using IgA as a binary variable ("high IgA" yes/no representing the highest tertile of IgA compared to tertiles 1 and 2 combined and "low IgA" yes/no representing the lowest IgA tertile compared to tertiles 2 and 3 combined) using Pearson Chi-square test. Crude associations between IgA and *C. difficile* colonization were also tested using linear regression (log IgA) and logistic regression models. To assess the potential confounding or effect modification by prenatal and postnatal factors including sex of the infant, birthmode, breastfeeding, maternal age, parity, maternal smoking, maternal weight status, maternal allergy status, exposure to household pets and antibiotic use on the associations between IgA and *C. difficile*, models were adjusted for each covariate separately. Variables were retained in final models at a p value of 0.05 or if they caused a ≥10% change in the estimate for *C. difficile* colonization. Covariates that did not improve the model fit or were correlated with other variables in the model were excluded. Smoking during pregnancy was not included in linear or logistic models as too few women smoked (only 3 of 46) and estimates were therefore unreliable. All analysis was conducted using IBM SPSS version 22. The study was approved by the University of Alberta, University of British Columbia and University of Manitoba Human Research Ethics Boards.

3. Results

Fecal IgA was measured in 47 infants from the Vancouver and Winnipeg sites of the CHILD study (Table 1). Median IgA was 14.28 μg per g protein (IQR 7.13–30.19) in fecal samples obtained from these infants (Table 2). Fecal IgA levels were higher in breastfed infants and those infants from primiparous mothers (Table 2). Over half (53%) of the infants were colonized by *C. difficile*. Median IgA was numerically lower in infants colonized with *C. difficile* (10.9 μg versus 25.5 μg per g protein; p = 0.18; Fig. 2). A lower proportion of infants with IgA in the highest tertile were colonized with *C. difficile* (31.3% versus 64.5%, p = 0.03; Fig. 3). There was no association between having IgA in the lowest tertile and *C. difficile* colonization (data not shown). *C. difficile* colonization was more common in non-breastfed infants and those born to younger mothers (Table 2). Infants born vaginally and those exposed to household pets were also more likely to be colonized although this did not reach statistical significance. No statistically significant differences in *C. difficile* colonization were found according to sex, maternal allergy, maternal smoking, maternal weight status or parity (Table 2). Data on the administration of antibiotics to mothers during birth, independent of delivery mode (restricted to those who did not have a cesarean section), and to infants before 3 months of age was only available in a subset (n = 14 and n = 17 respectively). There was some evidence that IgA levels were lower in

Table 1
Infant characteristics.

	N	%
Sex		
Male	22	46.8
Female	25	53.2
City of Birth		
Winnipeg	19	40.4
Vancouver	28	59.6
Mode of Delivery		
Vaginal	32	68.1
Elective cesarean	6	12.8
Emergency cesarean	9	19.1
Breastfeeding status (N = 46)		
Exclusive	21	45.7
Partial	13	28.3
None	12	26.1
Colonized with *C. difficile*		
Yes	25	53.2
No	22	46.8
Gestational age (weeks) ± SD (N = 43)	39.4 ± 1.6	
Birth weight (g) ± SD	3503 ± 436	
Age at fecal sample collection (months) ± SD	3.9 ± 0.58	

N = 47 unless otherwise indicated. SD, standard deviation.

Table 2
Fecal IgA levels and *C. difficile* colonization according to infant pre and postnatal exposures.

	N	(%)	Fecal IgA (μg/g total protein)			High fecal IgA[b]		*C. difficile* colonization	
			Median	(IQR)	p	N (%)	p	N (%)	p
Overall	47		14.28	(7.13–30.19)				25 (53.2)	–
Breastfeeding status[a]									
Exclusive	21	(46)	25.14	(7.33–38.39)	**0.03**	10 (47.6)	**0.03**[c]	7 (33.3)	**<0.01**[c]
Partial	13	(28)	14.28	(9.31–29.34)		5 (38.5)		7 (53.8)	
None	12	(26)	9.34	(4.24–16.28)		1 (8.3)		10 (83.3)	
Breastfed									
Yes (partially or exclusively)	35	(75)	23.11	(8.30–36.68)	**<0.01**	15 (42.9)	**0.03**	15 (42.9)	**0.02**
No	12	(26)	9.34	(4.24–16.28)		1 (8.3)		10 (83.3)	
Sex									
Male	22	(47)	19.74	(8.23–27.18)	0.47	9 (40.9)	0.35	11 (50.0)	0.68
Female	25	(53)	12.64	(6.84–26.92)		7 (28.0)		14 (56.0)	
Maternal age (years)[a]									
<30	12	(28)	10.93	(9.04–27.38)	0.47	4 (33.3)	0.74[c]	10 (83.3)	**0.04**[c]
30–35	14	(33)	22.85	(8.28–38.99)		7 (50.0)		5 (35.7)	
>35	17	(40)	21.27	(6.22–27.69)		5 (29.4)		7 (41.2)	
Maternal allergy or asthma[d]									
Yes	20	(43)	13.65	(8.32–31.07)	0.55	6 (30.0)	0.62	12 (60.0)	0.42
No	27	(57)	14.28	(5.76–30.19)		10 (37.0)		13 (48.1)	
Maternal smoking in pregnancy[a]									
Yes	3	(7)	8.28	(6.32–16.71)	0.41	–	0.21	3 (100.0)	0.1
No	43	(94)	14.28	(7.13–32.67)		15 (34.9)		22 (51.2)	
Maternal Weight Status[a]									
Normal (BMI<25)	24	(62)	12.34	(6.89–38.69)	1.00	9 (37.5)	0.79	13 (54.2)	0.96
Overweight or Obese (BMI 25+)	15	(39)	21.27	(10.93–27.31)		5 (33.3)		8 (53.3)	
Birth mode									
Vaginal	32	(68)	13.46	(6.89–35.24)	0.84	12 (37.5)	0.47	20 (62.5)	0.06
C-section	15	(32)	16.36	(7.78–26.35)		4 (26.7)		5 (33.3)	
Parity									
Multiparous	21	(45)	8.23	(4.48–25.14)	**0.04**	6 (27.3)	0.36	10 (45.5)	0.32
Primiparous	26	(55)	22.19	(10.39–34.30)		10 (40.0)		15 (60.0)	
Household pets									
Yes	28	(60)	10.45	(6.31–26.03)	0.14	7 (25.0)	0.11	18 (64.3)	0.06
No	19	(40)	23.35	(8.94–38.09)		9 (47.4)		7 (36.8)	
Maternal antibiotics[a]									
Yes	2	(14)	3.59	(2.92–4.25)	**0.05**	0 (0.0)	1.00	1 (50.0)	1.00
No	12	(86)	15.89	(6.89–26.03)		3 (25.0)		8 (66.7)	
Infant antibiotics before 3 months[a]									
Yes	2	(12)	28.13	(26.92–29.34)	0.13	2 (100.0)	**0.04**	1 (50.0)	1.00
No	15	(88)	9.31	(5.60–18.82)		2 (13.3)		9 (60.0)	

Comparisons of median fecal IgA by non-parametric Mann–Whitney U test (2 groups) or Kruskal–Wallis test (3 or more groups). Comparisons of high fecal IgA by Chi-square test. Fecal IgA, *C. difficile* colonization and breastfeeding status measured at mean age of 4 months. BMI, Body Mass Index (weight kg/height m^2). IQR Interquartile range. Bold denotes p value <0.05.

[a] Missing data. Maternal antibiotics (during the birth event) restricted to vaginal deliveries.

[b] High IgA = highest fecal IgA tertile versus other tertiles.

[c] Test for linear trend.

[d] Maternal current allergy or asthma.

infants from mothers given antibiotics during the birth event (p = 0.04; Table 2). Exposure to antibiotics maternally or during the first 3 months of life was not associated with *C. difficile* colonization (Table 2).

We did not find a linear association between fecal IgA concentration and *C. difficile* colonization. In unadjusted analysis, odds of colonization with *C. difficile* was reduced by 75% (OR 0.25 95% CI 0.07, 0.91 p = 0.04; Table 3) among infants with IgA in the highest tertile compared to those in the other tertiles. Following adjustment for breastfeeding, birth mode and parity this association was even stronger (aOR 0.17, 95% CI 0.03, 0.94, p = 0.04; Table 3). In this model, infant fecal *C. difficile* colonization was significantly less likely following breastfeeding and cesarean section delivery. There was no evidence of effect modification by any covariates.

4. Discussion

Consistent with more recent patterns of infant gut microbial composition in Western countries [3], over half of our sample of full-term infants from 2 urban centres in Canada were colonized by *C. difficile* at 3–4 months of age. Infant gut colonization by *C. difficile* is posited to be a biomarker for a less robust gut microbial ecosystem, marked by reduced abundance of

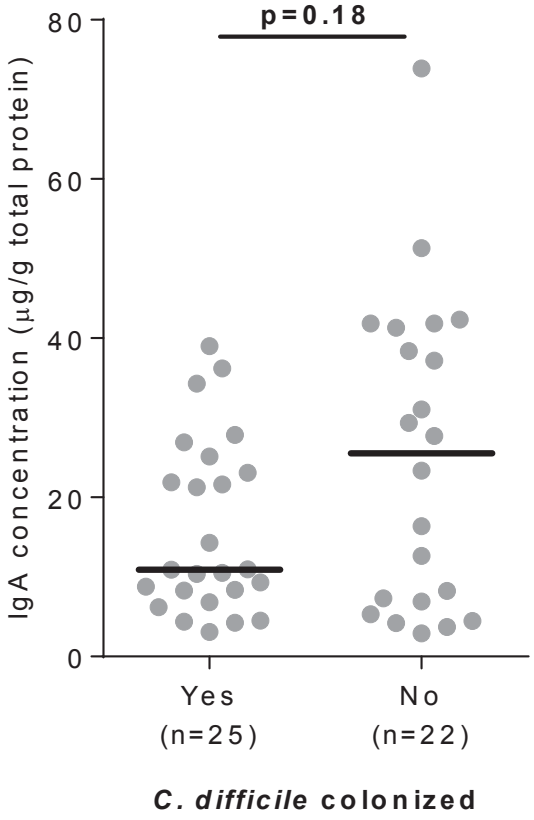

Fig. 2. Fecal IgA concentration according to *C. difficile* colonization. N = 47. Bars represent median values. Comparison by Mann Whitney Test. Fecal IgA and *C. difficile* colonization measured at mean age of 4 months.

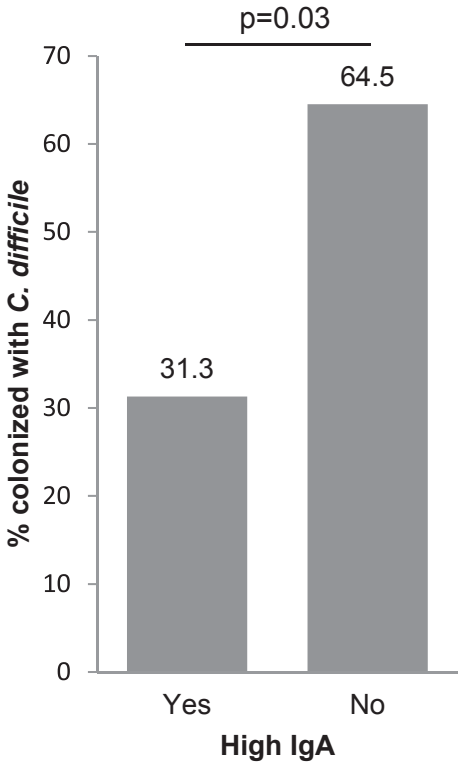

Fig. 3. *C. difficile* colonization according to fecal IgA. Comparison by Chi square test. High IgA, highest IgA tertile.

bifidobacteria, lactobacilli or *Bacteroides* spp [7,8] and lower resistance to pathogens. Colonization was 83% less likely with fecal IgA levels in the highest tertile. This finding was independent of breastfeeding status, maternal parity and birth mode, among which breastfeeding was the chief determinant of fecal IgA concentrations. In our previous report [21], infant fecal IgA levels varied by maternal and infant factors such as parity and infant sex, alone or in interaction with breastfeeding status. In the current study, only maternal parity predicted *C. difficile* colonization of the infant gut, independent of fecal IgA, with a greater risk of carriage seen in firstborn infants. The parity association is compatible with evidence that first born infants have less rapid gut colonization with lactobacilli and *Bacteriodes* species after birth [7], placing them at greater risk for lowered colonization resistance.

C. difficile is becoming a common pathogen in infant diarrhea [24]. Breakey et al. showed that higher breastmilk IgA levels were associated with lower gastrointestinal infection rates in nursing infants [25]. In our study, *C. difficile* colonization was also reduced in breastfed infants. In the fully adjusted model, the association between *C. difficile* detection and fecal IgA levels remained statistically significant, as did its independent association with breastfeeding status. Certainly, secretory IgA plays an important role in the immune exclusion of pathogens [11,12] and in shaping gut microbial composition [17,18]. Breast milk also contains other components with antibactierial properties including oligosaccharides, which can neutralize *C. difficile* by binding toxins produced [26]. Since these data were cross-sectional, a mediation analysis with temporal assessment of fecal IgA levels versus *C. difficile* colonization would be required to distinguish a causal role for fecal IgA in reducing *C. difficile* colonization from its role as a marker for other breastmilk bioactive agents.

C. difficile colonization, soon after birth or later in infancy, is more common following cesarean section delivery [2,27]. We observed the opposite association in our study, which may simply be a consequence of greater instrumentation during vaginal delivery or maternal antibiotic prophylaxis for group B *Streptococcus* in the group of infants born vaginally since this association was independent of breastfeeding status [23]. The more frequent administration of intrapartum antibiotics (during vaginal delivery) in nulliparous women may also explain some of the observed association between maternal parity and *C. difficile* colonization of infants [28]. Given that the parity and *C. difficile* association was independent of fecal IgA levels, it is also plausible that greater immaturity of innate immunity in firstborn infants may reduce colonization resistance and promote *C. difficile* colonization [29].

The main limitation of the study is sample size which will have affected the precision of our estimates of the association between IgA and *C. difficile*. In addition, although we were able to adjust for a number of important factors we cannot rule out residual confounding by other unmeasured variables. Due to the large amount of missing data on antibiotic exposure and limited evidence of the confounding effects of antibiotics in

Table 3
Likelihood of *C. difficile* fecal colonization in infants according to fecal IgA, adjusted and unadjusted for pre and postnatal factors.

	Unadjusted OR (95% CI)	Adjusted for breastfeeding aOR (95% CI)	Fully adjusted aOR (95% CI)
High IgA[a]	0.25 (0.07–0.91)	0.36 (0.09–1.38)	0.17 (0.03–0.93)
Breastfed	0.15 (0.03–0.79)	0.20 (0.04–1.13)	0.10 (0.01–0.86)
Cesarean section	0.30 (0.08–1.09)	–	0.11 (0.02–0.63)
Primiparous	1.5 (0.47–4.77)	–	5.00 (0.97–25.68)

OR, odds ratio. CI, confidence interval. Estimated by logistic regression using binary variable of *C. difficile* colonized. All variables were tested as binary variables (yes/no). Fecal IgA, *C. difficile* colonization and breastfeeding status measured at mean age of 4 months.

[a] High IgA = highest fecal IgA tertile versus other tertiles.

the association between fecal IgA and *C. difficile* colonization, we did not include this variable in our models. However, exposure to antibiotics, especially maternal intrapartum antibiotics [3], is one variable that will be important to include in future studies due to its significant impact on gut microbiota. In addition, measurements of IgA and *C. difficile* were obtained at only one time point when infants were on average 4 months old which limits the ability to infer causal relationships or directionality of the association. Our study also only explored the effect of one specific microbe on fecal IgA. *C. difficile* was specifically chosen due to its association with atopic disease independent of other components of the microbiome and because it is a marker for colonization resistance or delayed gut microbiota maturation [5,6]. The abundance measures reported by qRT-PCR are also far more robust than the semi-quantitative abundance data that can be mined from next generation sequencing profiles [30]. However, it will be important to study how other species, and the microbial community as a whole, are associated with gut immune development in future studies. Finally, our assay measured total IgA, based on the assumption that IgA found in fecal samples represents secretory IgA.

Our study is the first to provide evidence of an association between early gut immune development (fecal IgA) and colonization with *C. difficile,* an important source of *C. difficile* carriage in the community and a gut pathogen associated with later development of allergic disease [27]. Future research by our group will further explore associations between this early life intestinal immune marker, gut microbiota development and later child health including allergic disease.

Conflicts of interest

None.

Acknowledgments

The authors would like to thank Andrea Hill and Susan Goruk, University of Alberta for the analysis of fecal IgA. We are grateful to all the families who took part in this study, and the whole CHILD team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses. CHILD study investigators include: Sears MR, (Director), McMaster University; Subbarao P (co-Director), The Hospital for Sick Children; Allen R, Simon Fraser University; Anand SS, McMaster University; Becker AB, University of Manitoba; Befus AD, University of Alberta; Brauer M, University of British Columbia; Brook JR, University of Toronto; Chen E, Northwestern University, Chicago; Cyr M, McMaster University; Daley D, University of British Columbia; Dell S, Sick Children's Hospital; Denburg JA, McMaster University; Elliott S, University of Waterloo; Grasemann H, Sick Children's Hospital; HayGlass K, University of Manitoba; Hegele R, Sick Children's Hospital; Holness DL, University of Toronto; Lou WYW, University of Toronto; Kobor MS, University of British Columbia; Kollman TR, University of British Columbia; Kozyrskyj AL, University of Alberta; Laprise C, Université du Québec à Chicoutimi; Larché M, McMaster University; Macri J, McMaster University; Mandhane PM, University of Alberta; Miller G, Northwestern University, Chicago; Moqbel R (deceased), University of Manitoba; Moraes T, Sick Children's Hospital; Paré PD, University of British Columbia; Ramsey C, University of Manitoba; Ratjen F, Sick Children's Hospital; Sandford A, University of British Columbia; Scott JA, University of Toronto; Scott J, University of Toronto; Silverman F, University of Toronto; Takaro T, Simon Fraser University; Tang P, University of British Columbia; Tebbutt S, University of British Columbia; To T, Sick Children's Hospital; Turvey SE, University of British Columbia.

This research was specifically funded by the CIHR Canadian Microbiome Initiative (Grant No. 227312). The Canadian Institutes of Health Research (CIHR) and the Allergy, Genes and Environment (AllerGen) Network of Centres of Excellence provided core support for the CHILD study.

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