Environment International 142 (2020) 105881

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Natural environments in the urban context and gut microbiota in infants

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ARTICLE INFO

Handling Editor: Shoji Nakayama

Keywords:
Natural environments
Urban
Diversity
Gut microbiota
Infants

ABSTRACT

The biodiversity hypothesis that contact with natural environments (e.g. native vegetation) and biodiversity, through the influence of environmental microbes, may be beneficial for human commensal microbiota has been insufficiently tested. We aimed to study the association between living near natural environments in the urban context, and gut microbiota diversity and composition in young infants. Based on data linkage between the unique Urban Primary Land and Vegetation Inventory (uPLVI) for the city of Edmonton and 355 infants in the CHILD Cohort Study, infant exposure to natural environments (any and specific types, yes/no) was determined within 500 m and 1000 m of their home residence. Gut microbiota composition and diversity at age 4 months was assessed in infant fecal samples. Adjusted for covariates, we observed a reduced odds of high microbial alpha-diversity in the gut of infants exposed to any natural environment within 500 m [Shannon index aOR (95%CI) = 0.63 (0.40, 0.98) and Simpson index = 0.63 (0.41, 0.98)]. In stratified analyses, these associations remained only among infants not breastfed or living with household pets. When doubly stratifying by these variables, the reduced likelihood of high alpha-diversity was present only among infants who were not breastfed and lived with household pets [9% of the study population, Shannon index = 0.07 (0.01, 0.49) and Simpson index = 0.11 (0.02, 0.66)]. Differences in beta-diversity was also seen (p = 0.04) with proximity to a nature space in not breastfed and pets-exposed infants. No associations were observed among infants who were fully formula-fed but without pets at home. When families and their pets had close access to a natural environment, Verrucomicrobiales colonization was reduced in the gut microbiota of formula-fed infants, the abundance of Clostridiales was depleted, whereas the abundance of Enterobacteriales was enriched. Our double-stratified results indicate that proximity to a natural environment plus pet ownership has the capacity to alter the gut

Abbreviations: CHILD Cohort Study, Canadian Healthy Infant Longitudinal Development Cohort Study; uPLVI, Urban Primary Land and Vegetation Inventory; DEV, developed (anthropogenic non-vegetated); MOD, modified vegetated; NAT, natural non-vegetated; NAW, naturally wooded; NNW, naturally non-wooded; WET, wetland; OTU, operational taxonomic unit

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https://doi.org/10.1016/j.envint.2020.105881
Received 26 March 2020; Received in revised form 27 May 2020; Accepted 7 June 2020
Available online 28 June 2020

microbiota of formula-fed infants. Further research is needed to replicate and better interpret these results, as well as to understand their health consequences.

1. Introduction

Global loss of biodiversity, in tandem with loss of diversity in human microbiota, has occurred in parallel with the rise of immune and inflammatory disorders and processes, particularly in Western society (Hanski et al., 2012; Rook, 2013; Ruokolainen, 2017; Tasnim et al., 2017). Natural environments, be it native vegetation, soil or water, and their microbial ecosystems are being posited as interventions to enrich the human microbiome, balance the immune system, and guard against allergy and inflammatory disorders (Haahtela, 2019). Under this biodiversity hypothesis, the preservation or re-introduction of natural environments in urban centres might help counterbalance the impacts of urbanization on human health through the influence of environmental microbes on human microbiota (Flies et al., 2017; Ileka-Priouzeau et al., 2015; Tasnim et al., 2017). This approach to utilizing the biodiversity of nature, as one of 'nature's gifts,' to modify human microbiota is being regarded by the WHO are the way forward towards restoring, maintaining and enhancing human health and well-being (World Health Organization and Secretariat of the Convention on Biological Diversity, 2015). Others acknowledge that the biodiversity hypothesis is an interesting concept but one which requires scrutiny to better understand the complexity of microbial inputs from the environment on the developing infant microbiome and immune system (von Mutius, 2018).

To date, few studies have tested the nature biodiversity hypothesis and its implications for human health. In a first study on the impact of environmental biodiversity within urban neighbourhoods (N = 118), adolescents with allergic conditions, compared to healthy individuals, were more likely to live in homes surrounded by lower environmental biodiversity and had significantly lower diversity of gamma-Proteobacteria on their skin (Hanski et al., 2012). In another study that included a large number of children and adolescents (N = 1044), an increasing percentage of forest and agricultural land cover within 2–5 km of the residential home was inversely associated with allergic sensitization in children (Ruokolainen et al., 2015). The relative abundance of Proteobacteria increased significantly on the skin of healthy individuals along the gradient from built to green environments. Although not related to atopic sensitization status, skin microbial diversity of preschool children attending a nature-based day care was observed to be higher than children attending city-centre day cares (Lehtimäki et al., 2018). Other studies also find rural–urban differences but in the opposite direction, with significantly lower skin microbial diversity among rural as compared to urban adolescents (Lehtimäki et al., 2017). In the only study of infancy to date, both the amount of farmland and forest cover were found to differentiate the presence of the Proteobacteria, *Acinetobacter*, on the skin and nares of 6-month old infants (Ruokolainen et al., 2020). Much of the previous research relied on greenness measures derived from satellite imagery where generalized land cover or the normalized difference vegetation index (NDVI) were used in assigning exposure to the vegetated environment (Cusack et al., 2018; Dadvand et al., 2014; Liu et al., 2019; Mhuireach et al., 2016), which did not differentiate between natural and semi-natural (i.e. anthropogenic maintained green spaces in parks/sports fields, residential, and agricultural areas) vegetated urban spaces.

To our knowledge, there are no existing studies assessing the relationship between natural environments in the urban context and the human gut microbiome during the early years of life. The first three years of life are key in the establishment of the adult gut microbiota,

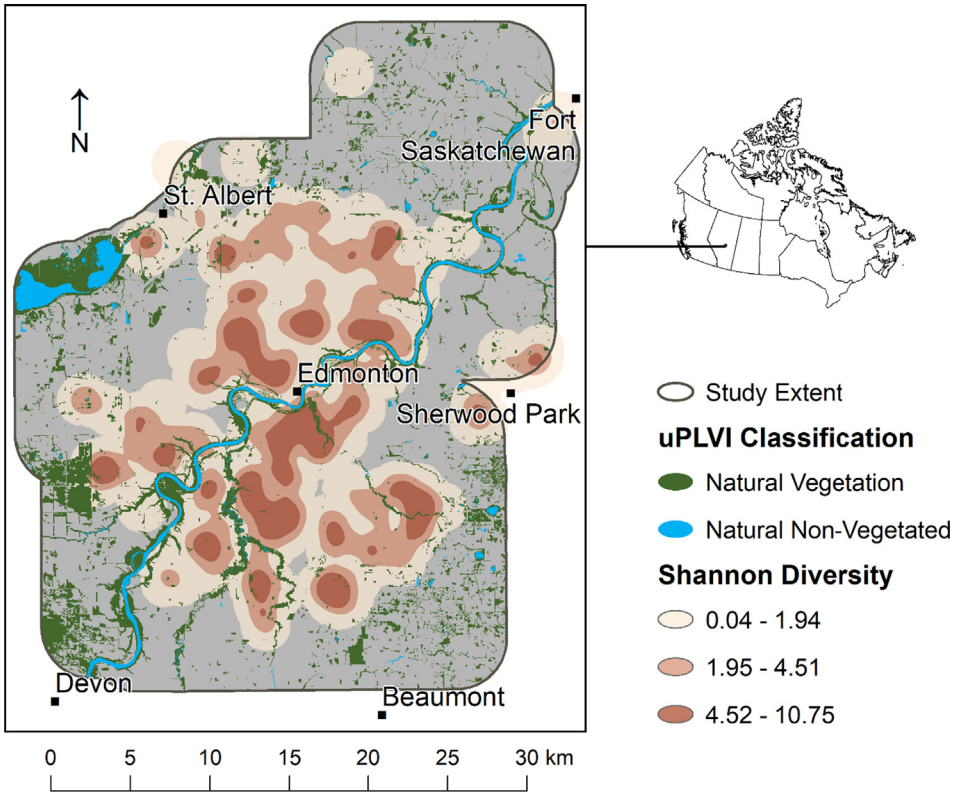

Fig. 1. Distribution of the natural land types and density of infant gut microbial samples having low to high Shannon diversity.

and its study is of particular interest because gut microbes tune local and systemic immune responses to confer protective immunity against pathogens, while simultaneously maintaining immune tolerance toward commensals (Tasnim et al., 2017). The aim of the present study was to explore the associations between living near natural environments in the urban context and gut microbial diversity and composition in infants at the age of 4 months.

2. Materials and methods

2.1. Study population

We accessed data from the Edmonton, Alberta site of the CHILD (Canadian Healthy Infant Longitudinal Development) birth cohort study (www.childstudy.ca). Study approval was obtained from the University of Alberta Research Ethics Board; the CHILD Cohort Study was approved by the Hamilton Integrated Ethics Board (certificate number 07–2929). Parents provided consent at the time of recruitment between 2009 and 2012. Of 765 Edmonton site infants, 143 lived outside the area of the Edmonton vegetation map, leaving 355 infants with complete gut microbial profiles at age 4 months available for analysis.

2.2. Potential exposure to natural environments in the urban context

We used the Urban Primary Land and Vegetation Inventory (uPLVI) of the city of Edmonton (City of Edmonton (Canada), 2015) to define potential exposure to natural and anthropogenic environments in the urban context. The original boundaries, mapped via detailed photo-interpretation at a 1:20,000 scale (30 cm digital resolution), were converted to a raster with 10 m cell sizes to retain detail. There were six land classes defined as: developed non-vegetated with anthropogenic origin (DEV), modified vegetated with anthropogenic origin (MOD), natural non-vegetated naturally occurring features (NAT; blue spaces such as a lake or a river (Grellier et al., 2017)), naturally wooded vegetated having a greater than or equal to 6% tree cover (NAW), naturally non-wooded vegetated having a $< 6\%$ tree cover (NNW), and wetland vegetated with minimum hygric moisture regimes (WET). Fig. 1 shows the distribution of the natural land types in Edmonton. For the mapping and spatial analysis of natural vegetation in proximity to the CHILD Cohort Study participant residences, we used ArcGIS software (Esri, 2017). The 6-character postal code, which represents an address (e.g. a single building) or group of addresses (e.g. a city block as one side of a street between two intersecting streets) in an urban area (Canada Post, 2019), of each infant residence was assigned to its corresponding geographic coordinates. Then, we calculated 500 m and 1000 m buffer zones around each residential location (Fig. 2), after which the percentage of each land type within a buffer zone was determined and assigned to the location of residence. Because the distribution of the NAT, NAW, NNW and WET measures was skewed towards 0 (see the supplemental material, Fig. A1), a binary variable was created for each based on the respective median. A binary variable for exposure to any natural environment was also created by summing the percentage of NAT, NAW, NNW and WET within a buffer zone, then defining exposure to any natural environment (no vs. yes) if the sum of these four land types differed from zero (i.e. no $= 0\%$ of exposure, and yes $\neq 0\%$ of exposure to any natural environment). This variable was the main exposure variable used in our study, while the variables of exposure to specific natural environments were only used in a sensitivity analysis. We did not consider MOD in the exposure variable to any natural environment because we were interested in the effects of natural environments that excluded semi-natural environments (e.g. vegetated areas with anthropogenic origin). Semi-natural environments have a different soil microbial ecology due to grass cover, non-native vegetation, maintenance practices (e.g. use of pesticides) and more intensive use by humans. However, and as explained below, we did control our models for exposure to MOD in a sensitivity analysis.

2.3. Gut microbiota analysis

Fecal samples of infants were collected at the age of 4 months (mean = 4.2, range = 2.6 to 7.5 months) using a standard protocol during a planned home visit. Methods of sample collection, DNA extraction and amplification, 16S rRNA sequencing and taxonomic classification have been previously described (Tun et al., 2017). In summary, DNA was extracted from fecal samples stored at −80 °C and the V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR using universal primers and sequenced by the Illumina Miseq platforms. Taxonomic assignment of sequences was achieved using the RDP classifier constrained by the GREENGENES reference database (v13.8). With the QIIME pipeline, relative abundance of bacterial OTUs (Operational Taxonomic Units) was summarized at the phylum, order and family levels. OTUs with a relative abundance below 0.0001 were excluded; data were rarefied to 13,000 sequences. A PCA plot of microbial beta-diversity at the order level of composition was constructed in R with the "stats" package to identify clustering of infant gut microbiota by natural environment exposure. Microbial species richness or number of different species was assessed by the Chao1 index. Alpha-diversity (microbial species richness and evenness) was measured with Shannon and Simpson diversity indices, with the Shannon index placing more weight on species richness and the Simpson index placing more weight on species evenness (for both indices, a higher value indicates higher diversity). All equations for alpha-diversity are reported in Table A. As per Fig. 1, the density of infant fecal samples with low to high Shannon microbiota diversity was mapped in relation to areas of natural vegetation. Chao1, Shannon and Simpson indices were calculated to represent all microbiota in a sample, as well as microbiota categorized at the phylum level (i.e. Bacteroidetes species richness). Based on median values, binary variables were created for Chao1, Shannon and Simpson indices (i.e. high versus low diversity, as the reference category) and for OTU relative abundance. We paid particular attention to

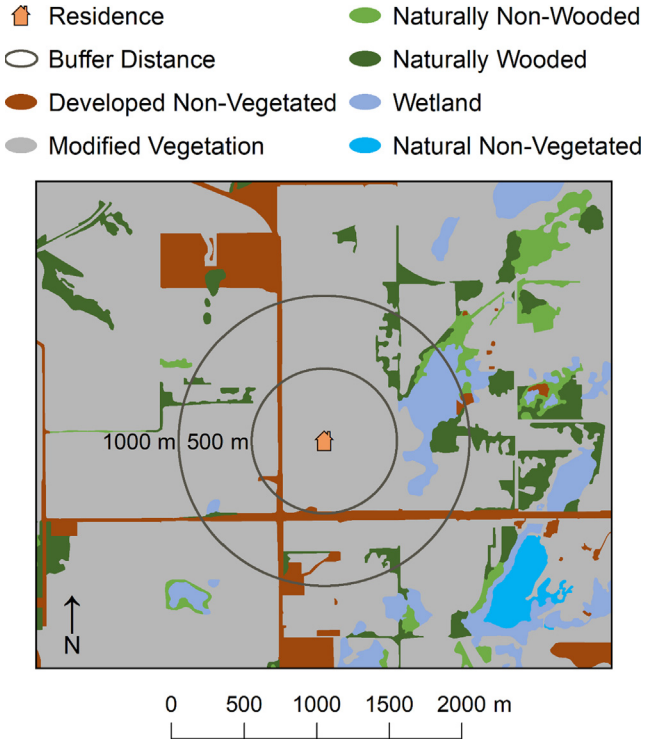

Fig. 2. Example of the buffers used (500 m and 1000 m) and the information obtained based on residential address of the participants.

bacterial OTUs observed to be prevalent in soil and the human gut (Delgado-Baquerizo et al., 2018; Shin et al., 2015): Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes.

2.4. Statistical analysis

Pairwise comparisons of beta-diversity between infants stratified by nature exposure, feeding status and pets exposure were conducted with the PERMANOVA tests using the Bray-Curtis dissimilarity metric. We also employed logistic regression to determine associations with binary measures of gut microbiota that were, based on previous literature (Azad et al., 2015), adjusted for four variables: infant age, season at time of fecal sample collection [summer yes or no (Hui et al., 2019)], breastfeeding status (formula-fed, partially or exclusively breastfed), and birth mode-antibiotic use (cesarean section with maternal intrapartum antibiotic prophylaxis, vaginal birth with intrapartum antibiotic prophylaxis, vaginal birth and no intrapartum antibiotics). Although associated with exposure to a natural environment (Table 1), maternal age and education level were correlated with breastfeeding status ($p < 0.001$ for maternal university education) and birth mode ($p < 0.02$ for maternal age), and excluded from statistical models to prevent over-adjustment.

Reduced gut microbial diversity is an expected biological phenomenon in the 4-month old breastfed infant (Azad et al., 2013), so it was also important to test associations in models stratified by infant feeding status. We also stratified models according to presence of pets at home (no, yes) since pet ownership is a strong determinant of gut microbiota composition (Tun et al., 2017) and of greater use of natural environments surrounding households (Zijlema et al., 2019). Finally, given that other exposures in the urban context can potentially influence gut microbiota [e.g. air pollution (Vallès and Francino, 2018)], we assessed whether associations between exposure to natural environments in the city context, and gut microbiota composition and diversity varied according to the levels of urbanization within 500 m and 1000 m of the residence (as per tertiles of the variable DEV). Finally, we conducted additional sensitivity analyses: (i) to test whether the inclusion of modified vegetated with anthropogenic origin (MOD) in the model (binary variable based on the median, see supplemental material Fig. A2) or the stratification of the models by MOD affected the associations between exposure to any natural environment and gut microbiota indicators; (ii) using the 1000 m buffer, instead of the 500 m; and (iii) to assess the effects of each specific type of natural environment (NAT, NAW, NNW and WET). We used STATA 14 to conduct our analyses (StataCorp, 2016) and carry out multiple imputation prior to statistical modelling to avoid loss of participant information (Royston, 2005).

3. Results

The main characteristics of infants exposed to any natural environment (53.5% when considering the 500 m buffer) versus those who were not (46.5%) were: breastfeeding status (61.4% exclusively breastfed in the exposed group vs. 43.3% in the non-exposed group), maternal post-secondary degree (78.1% vs. 65.4%, respectively) and maternal age (mean of 32.0 vs. 30.7 years, respectively) (Table 1). Shannon and Simpson diversity values above the median were more prevalent among non-exposed infants (57% and 56.4%, respectively) than exposed (43.7% and 44.2%; p-value for differences between exposure groups = 0.01 and 0.02, respectively). No statistically significant differences were observed for Chao1 species richness of gut microbiota according to infant proximity to a natural environment

Table 1
Distribution of variables for the total study population (N = 355) and by exposure to any natural environment[a] within a 500 m buffer zone.

	%, mean (SD) or median	Non-exposed (46.5%)	Exposed (53.5%)	p-value for differences between exposure groups[a]
Sex (% males)	49.7	50.3	49.2	0.84
Feeding type (%)				
No breastfeeding	18.1	23.2	13.8	0.002
Partial breastfeeding	28.9	33.5	24.9	
Exclusive breastfeeding	53.0	43.3	61.4	
Type of birth & antibiotics use at birth (%)				
Cesarean section & antibiotics	23.6	25.6	21.8	0.69
Vaginal birth & antibiotics	25.9	25.6	26.1	
Vaginal birth & no antibiotics	50.6	48.8	52.1	
Ethnicity (%)				
White	75.5	75.9	75.1	0.44
Asian	12.0	13.6	10.6	
Other	12.5	10.5	14.3	
Pets at home after birth (% yes)	50.3	51.3	49.5	0.74
Older siblings (% yes)	49.2	46.6	51.3	0.38
Maternal university degree (% yes)	50.7	41.2	58.9	0.001
Household income (%)				
< 50.000$	11.8	14.8	9.3	0.15
50.000$ − 99.999$	35.8	37.4	34.4	
≥100.000$	45.3	39.4	50.3	
Prefer not to say	7.1	8.4	6.0	
MOD (modified vegetated with anthropogenic origin; median of coverage %)	12.2	10.1	14.4	< 0.001
Maternal BMI [mean (SD)]	25.9 (6.0)	24.7 (6.1)	25.5 (5.9)	0.10
Maternal age [mean (SD)]	31.3 (4.6)	30.7 (4.9)	32.0 (4.2)	0.004
Age at the time of fecal sample collection [mean (SD)]	4.2 (1.2)	4.1 (1.1)	4.2 (1.3)	0.33
Fecal sample collected during summer season (% yes)	31.0	29.1	32.6	0.47
Gut microbiota at age 4 months (median and % above the median)				
Chao1 species richness index	195.2 (49.9%)	197.3 (50.9%)	195.1 (49.0%)	0.50 (0.71)[b]
Shannon diversity index	3.1 (49.9%)	3.2 (57.0%)	3.1 (43.7%)	0.03 (0.01)[b]
Simpson diversity index	0.77 (49.9%)	0.80 (56.4%)	0.76 (44.2%)	0.04 (0.02)[b]

[a] Chi-square test for categorical variables, *t*-test for maternal age, Wilcoxon rank-sum test for maternal BMI (body-mass index), infant age at time of fecal sample collection and the Chao1, Shannon and Simpson indices.

[b] p-values for median differences between groups (and for the % above the median).

(Table 1).

An inverse association was found between exposure to natural vegetation within 500 m of the residential home and higher infant gut microbial diversity according to the Shannon [aOR (95%CI) = 0.63 (0.40, 0.98)] and Simpson indices [aOR (95%CI) = 0.63 (0.41, 0.98)]. No associations were observed with the Chao1 index [aOR (95%CI) = 0.97 (0.62, 1.54)] (Table 2). Adjustment of models for infant age, birth mode, breastfeeding status or season at time of fecal sample collection did not change these gut microbiota associations with exposure to any natural environment (Table 2). The results of stratified analyses are reported in Table 2. For all three variables tested (feeding type, household pets, and level of urbanization) the direction of the association with the Shannon and Simpson indices was the same, and aligned with findings from the whole study population. Although there was no evidence of effect modification ($p > 0.10$), we observed that the magnitude of exposure to any natural environment was stronger among infants who were formula-fed [aOR (95%CI) = 0.21 (0.06, 0.66) for Shannon diversity and 0.33 (0.11, 0.98) for Simpson diversity] as compared to those who were partially [0.91 (0.39, 2.12) and 0.77 (0.34, 1.75), respectively] or exclusively breastfed at that time [0.74 (0.39, 1.39) and 0.71 (0.38, 1.31), respectively].

Among infants living with pets in the same household (Table 2), we also found an inverse association between exposure to a natural environment and the Shannon index [aOR (95%CI) = 0.48 (0.25, 0.93)] or the Simpson index [aOR (95%CI) = 0.40 (0.21, 0.77)]. Associations were not found with gut microbiota diversity in the absence of household pets. Given these results, and the limited sample size of the study, we applied a two-level stratified analysis by feeding type and by presence of pets at home (Table 3). The reduced likelihood of having a high gut microbiota diversity with close proximity to natural vegetation was evident only among infants who were formula-fed and lived with household pets [9% of the study population, aOR (95%CI) = 0.07 (0.01, 0.49) for the Shannon index and 0.11 (0.02, 0.66) for Simpson index]. Similarly, a comparison of cut-off values for the diversity binary outcomes yielded lower medians in nature-exposed versus non-exposed infants of this stratum: 2.91 (inter-quartile range [IQR] 2.63–3.61) versus 3.84 (IQR 3.39–4.08), $p = 0.05$, for the Shannon index, and 0.71 (IQR 0.63–0.84) versus 0.86 (IQR 0.78–0.88), $p = 0.07$, for the Simpson index. These associations were not observed among formula-fed peers without pets at home (Table 3). Among infants partially or exclusively breastfed, there were no nature-gut microbiota associations in the presence or absence of pets at home. Whereas expected clustering by breastfeeding status was seen in the PCA plot (Fig. 3), separation by proximity to natural vegetation was less visible. However, tests of beta-diversity found statistical significance with exposure to a natural space among infants not breastfed and living with pets ($p = 0.04$).

Exposure to a natural environment within 500 m of the home was unrelated to the relative abundance (low vs. high) of the bacterial phyla, Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Table 4). Regarding gut microbial species richness and diversity within these four major phyla, we observed a statistically significant reduced likelihood of high Simpson diversity within the Proteobacteria phylum

Table 3
Associations between being exposed to natural environments within 500 m of the residential address and, Shannon and Simpson indices[a] in infant gut microbiota at age 4 months (N = 355) doubly stratified by feeding type and presence of pets at home.

Feeding type and presence of pets at home (subjects (%) within each category)	Shannon index[a] Adjusted[b] model aOR (95%CI)	Simpson index[a] Adjusted[b] model aOR (95%CI)
No breastfeeding		
No pets at home (N = 32, 9%)	0.62 (0.10, 3.91)	1.17 (0.22, 6.37)
Yes pets at home (N = 32, 9%)	0.07 (0.01, 0.49)	0.11 (0.02, 0.66)
Partial breastfeeding		
No pets at home (N = 43, 12%)	1.14 (0.26, 4.92)	1.60 (0.38, 6.76)
Yes pets at home (N = 60, 17%)	0.89 (0.28, 2.79)	0.48 (0.16, 1.51)
Exclusive breastfeeding		
No pets at home (N = 103, 29%)	0.83 (0.35, 1.95)	0.83 (0.36, 1.91)
Yes pets at home (N = 85, 24%)	0.58 (0.21, 1.59)	0.55 (0.21, 1.43)

[a] Higher Shannon or Simpson index values indicate higher microbial diversity, as measured by species richness and species evenness, with the Shannon index giving more weight to species richness and the Simpson index giving more weight to species evenness. Indices were dichotomized (low vs high, low being the reference category) based on the median.

[b] Age of infant at time of fecal sample collection, birth mode and antibiotic use (combined variable), season of fecal sample collection.

Table 2
Associations between being exposed to natural environments within 500 m of the residential address and high versus low Chao1, Shannon and Simpson[a] diversity in infant gut microbiota at age 4 months (N = 355), stratified by feeding type, presence of pets at home and level of urbanization.

	Chao1 index[a]		Shannon index[a]		Simpson index[a]	
	Crude model OR (95%CI)	Adjusted[b] model aOR (95%CI)	Crude model OR (95%CI)	Adjusted[b] model aOR (95%CI)	Crude model OR (95%CI)	Adjusted[b] model aOR (95%CI)
Whole study population	0.92 (0.61, 1.40)	0.97 (0.62, 1.54)	0.59 (0.38, 0.89)	0.63 (0.40, 0.98)	0.61 (0.40, 0.93)	0.63 (0.41, 0.98)
Stratified by feeding type[c]						
No breastfeeding	0.48 (0.17, 1.36)	0.38 (0.12, 1.19)	0.23 (0.07, 0.71)	0.21 (0.06, 0.66)	0.36 (0.13, 1.04)	0.33 (0.11, 0.98)
Partial breastfeeding	1.29 (0.59, 2.83)	1.27 (0.56, 2.90)	0.45 (0.43, 2.06)	0.91 (0.39, 2.12)	0.76 (0.36, 1.69)	0.77 (0.34, 1.75)
Exclusive breastfeeding	1.20 (0.66, 2.19)	1.14 (0.60, 2.19)	0.75 (0.41, 1.36)	0.74 (0.39, 1.39)	0.71 (0.39, 1.29)	0.71 (0.38, 1.31)
Stratified by pets at home[c]						
No	0.85 (0.47, 1.55)	0.79 (0.41, 1.52)	0.74 (0.41, 1.35)	0.80 (0.42, 1.52)	0.88 (0.48, 1.60)	0.96 (0.51, 1.79)
Yes	1.01 (0.56, 1.82)	1.27 (0.64, 2.52)	0.46 (0.25, 0.84)	0.48 (0.25, 0.93)	0.42 (0.23, 0.77)	0.40 (0.21, 0.77)
Stratified by level of urbanization[c,d]						
T1 (< 78% DEV)	0.63 (0.23, 1.74)	0.72 (0.23, 2.27)	0.34 (0.11, 1.03)	0.46 (0.14, 1.55)	0.44 (0.16, 1.26)	0.42 (0.14, 1.32)
T2 (78–89% DEV)	1.40 (0.68, 2.91)	1.35 (0.63, 2.93)	0.53 (0.25, 1.11)	0.47 (0.21, 1.04)	0.69 (0.33, 1.45)	0.63 (0.29, 1.37)
T3 (> 89% DEV)	0.38 (0.15, 0.90)	0.57 (0.22, 1.52)	0.56 (0.15, 1.27)	0.84 (0.35, 2.04)	0.48 (0.22, 1.10)	0.67 (0.28, 1.60)

[a] Higher Chao1 (species richness) index values indicate a greater number of different microbial species; higher Shannon or Simpson index values indicate higher microbial diversity, as measured by species richness and evenness, with the Shannon index giving more weight to species richness and the Simpson index giving more weight to species evenness. Indicators were dichotomized (low vs high, low being the reference category) based on the median.

[b] Age of the child at the time of fecal sample collection, breastfeeding, mode of delivery and antibiotic use (combined in one single variable), season of fecal sample collection. Adjustment only for whole study population.

[c] p-values for interaction between exposure to natural environments and the variables tested > 0.1.

[d] Level of urbanization was defined based on tertiles of the variable "developed non-vegetated with anthropogenic origin (DEV)" of the Urban Primary Land and Vegetation Inventory (uPLVI) of the city of Edmonton (City of Edmonton (Canada), 2015).

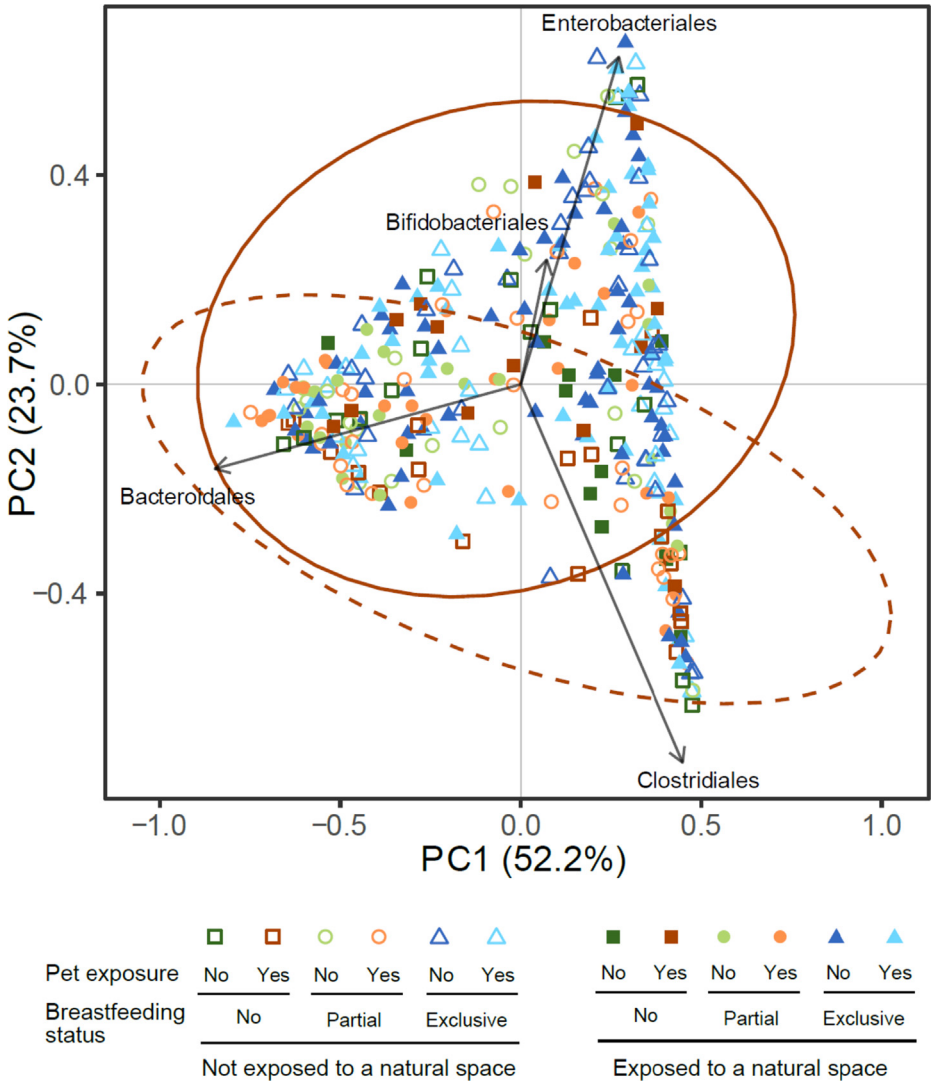

Fig. 3. Principal component analysis (PCA) for order-level microbiota in infant fecal samples at 4 months of age. The first two principal components, PC1 and PC2, were plotted. Exposure to a natural space, breastfeeding status and household pet ownership were denoted by different colours and shapes in solid or no fill. Vectors corresponding to the top 4 bacterial orders with the highest loadings are displayed. Two ellipses representing 95% confidence interval for the centroids of gut microbiota were drawn to highlight clusters for natural space proximity (yes/no) in non-breastfed infants living in homes with pets.

Table 4
Associations between being exposed to natural environments within 500 m of the residential address and, relative abundance of five phyla, and microbial diversity within each phylum in infant gut microbiota at age 4 months (N = 355).[a]

	Phylum abundance[b] Adjusted[d] model aOR (95%CI)	Chao1 index[c] Adjusted[d] model aOR (95%CI)	Shannon index[c] Adjusted[d] model aOR (95%CI)	Simpson index[c] Adjusted[d] model aOR (95%CI)
Proteobacteria	0.81 (0.52, 1.25)	1.14 (0.74, 1.76)	0.81 (0.53, 1.26)	0.63 (0.41, 0.97)
Actinobacteria[a]	1.17 (0.76, 1.81)	1.69 (1.09, 2.63)	0.70 (0.46, 1.09)	0.67 (0.43, 1.04)
Bacteroidetes[a]	1.08 (0.67, 1.75)	0.77 (0.48, 1.24)	0.68 (0.44, 1.04)	0.72 (0.47, 1.11)
Firmicutes	0.77 (0.49, 1.20)	1.34 (0.84, 2.13)	1.07 (0.66, 1.74)	1.03 (0.65, 1.63)
Verrucomicrobia	1.11 (0.61, 2.02)	[e]	[e]	[e]

[a] One infant had missing values for Actinobacteria Shannon and Simpson indices, and two infants had missing values for Bacteroidetes Shannon and Simpson indices.

[b] Relative abundance for each phylum was dichotomized based on the median (low vs high, low being the reference category).

[c] higher Chao1 (species richness) index values indicate a greater number of different species; higher Shannon or Simpson index values indicate higher microbial diversity, as measured by species richness and species evenness, with the Shannon index giving more weight to species richness and the Simpson index giving more weight to species evenness. Indices were dichotomized (low vs high, low being the reference category) based on the median.

[d] Age of infant at time of fecal sample collection, breastfeeding, birth mode and antibiotic use (combined variable), season of fecal sample collection.

[e] Verrucomicrobia only include one species.

[aOR (95%CI) = 0.63 (0.41, 0.97)] but a statistically significant increased likelihood of high Chao1 species richness within the Actinobacteria phylum [aOR (95%CI) = 1.69 (1.09, 2.63)] if infants were exposed to a natural environment (Table 4). Effect modification by breastfeeding status was found for the Chao1 index within the Bacteroidetes and Firmicutes species, such that higher species richness within both phyla was less likely among non-breastfed infants exposed to natural vegetation. No phylum-specific diversity associations with nature exposure were evident among infants partially or exclusively breastfed (data not shown). No effect modification was observed by presence of pets at home.

When stratified by exposure, household pets and breastfeeding status at the order level of bacterial classification (Fig. 4), the Verrucomicrobiales (namely *Akkermansia*, the only genus in this order) were more abundant in nature exposed than non-exposed infants across all feeding types, especially in formula-fed infant ($p < 0.06$). Compared to formula-fed infants living in homes without pets but with close proximity to a natural environment, the following trends were seen in formula-fed infants living in homes with pets and close access to a natural environment: lower microbial diversity, lower colonization rates with Verrucomicrobia (46% vs 26%, $p = 0.22$), lower abundance of Clostridiales (median 13.2% vs. 37.3%, $p = 0.08$) and higher abundance of Enterobacteriales (of Proteobacteria, median 23.9% vs. 10.8%, $p = 0.14$). Among all formula-fed infants exposed to household pets, proximity to a natural environment versus not differentiated their gut microbiota in terms of a lower abundance of Clostridiales (median 13.2% vs. 41.4%, $p = 0.01$), and a higher abundance of Enterobacteriales (median 23.9% vs. 8.3%, $p = 0.02$).

In our study population those infants classified as living within 500 m of any natural environment had a higher median proportion of urban green areas [i.e. modified vegetated with anthropogenic origin (MOD); median of 14.4% vs median of 10.1% in infants classified as non-exposed to natural environments, $p < 0.001$ for differences in the proportions]. We observed that the associations between exposure to any natural environment and gut microbiota diversity remained towards the same direction but lost statistical significance (e.g. aOR (95%CI) = 0.65 (0.41, 1.03) for Shannon and 0.69 (0.44, 1.08) for Simpson] once exposure to urban green areas was included in the model. When we stratified by levels of MOD (low and high exposure, based on the median of land coverage 12.2%), association between exposure to natural environments and Shannon remained among those highly exposed to MOD [0.50 (0.25, 1.00)], but not among those with low exposure to MOD [0.81 (0.43, 1.54)] (see supplemental material, Table B). In relation to other additional analyses, using the 1000 m buffer yielded similar results (data not shown), while assessing the effects by each specific type of natural environment (NAT, NAW, NNW and WET) provided non-statistically significant results, but towards the same direction as when considering all the four natural types together (data not shown).

4. Discussion

The natural environment is an ecosystem service provider that bridges public health and conservation strategies (World Health Organization and Secretariat of the Convention on Biological Diversity, 2015) with real potential to improve health through the human microbiome (Liddicoat et al., 2020). Appreciating this critical bridging role, but knowing that studies of the natural - wild - environment are limited (van den Bosch and Ode Sang, 2017) and those assessing impact on the gut microbiome at a critical age of development are non-existent, we undertook this study to evaluate the impact of living near natural environments in an urban context on the gut microbiota of 355 young infants. Our findings indicate that when 4-month old infants live near natural environments, their gut microbiota are less likely to have a high diversity of species. While seemingly counter-intuitive, it is important to point out that this gut microbial community structure is more typical

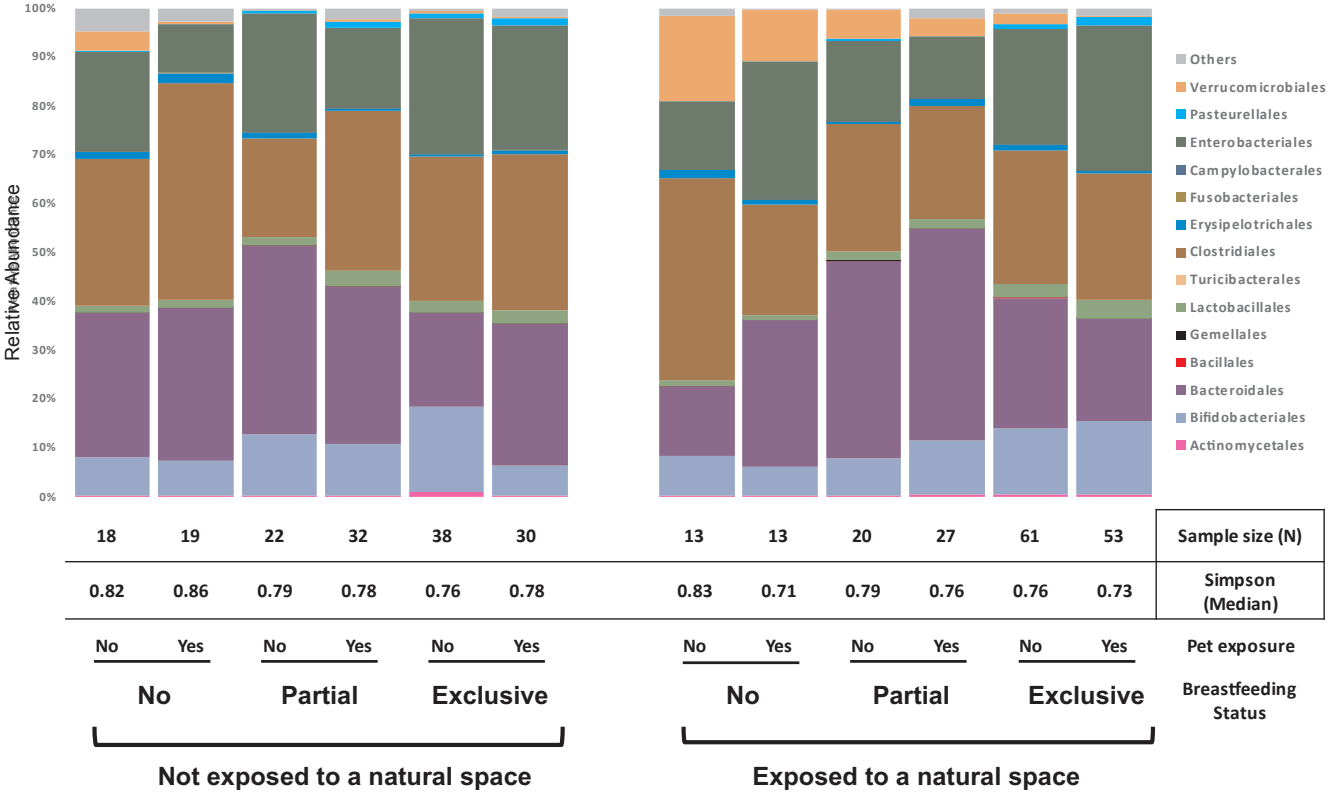

Fig. 4. Infant gut microbiota composition – at the order level – of the three feeding groups (no, partial, and exclusive breastfed) by exposure to pets and natural environment.

of a young breastfed infant (Forbes et al., 2018). Furthermore, and even within this small sample of infants, observed associations with nature were only apparent in infants who were concurrently exclusively formula-fed and exposed to pets at home.

To unpack our findings, let us first recap what is known about the impact of the natural environment on human microbiota. To date, the limited literature has been inconsistent, with one study suggesting that exposure to these environments, as measured by the percentage of plant species surrounding homes, increases skin microbial diversity in exposed adolescents (Hanski et al., 2012), another study reporting enrichment of skin Proteobacteria in children and adolescents following greater exposure to forest (Ruokolainen et al., 2015), and a third study noting a reduction of microbial diversity in the skin of adolescents surrounded by a natural environment, as defined by remotely-sensed land cover (Lehtimäki et al., 2017). However, these are studies of skin microbiota; gut microbiota, the subject of our study, are an entirely different microbial community. Body site differences in microbial communities are even more pronounced in young infants when, for example, Proteobacteria are highly present in the gut but less prevalent in skin (Parm et al., 2018).

Notable in our study is the finding of a stronger reduction in gut microbial diversity among formula-fed infants, while the same trend was not statistically significant among infants partially or exclusively breastfed. It is biologically plausible that any potential influence of a natural environment on infant gut microbiota is mitigated by the stronger influence of breastfeeding. Microbes associated with breast milk and maternal skin are more abundant in the gut of breastfed infants compared to formula-fed infants (Timmerman et al., 2017). In addition, we observed that having a pet also seemed to play a key role. Indeed, the associations between exposure to natural vegetation, and infant gut (alpha or beta) diversity or abundance of Enterobacteriales were observed only among infants fed formula and living in homes with pets. Formula-fed infants are susceptible to colonization by a wide range of microbiota that are able to digest the simple sugars and protein of formula (Chow et al., 2014; Marcobal et al., 2010). These ingredients in formula, or the gut microbial composition they promote, may attract the microbiota present in natural environments that are brought into the home by the family pet. The microbiota of dust in Canadian homes is reported to be dominated by Proteobacteria in the presence versus absence of pets, especially dogs (Konya et al., 2014). Further, the adoption of a dog has shown capacity to rapidly introduce rare bacterial taxa into home dust (Sitarik et al., 2018).

Without being able to characterize the microbiota of the surrounding natural environment or house dust, we hypothesize that reduced gut microbial diversity, specifically within the Proteobacteria, occurs because exposure to natural environments leads to overgrowth of specific microbes. This phenomenon has been seen with household pet ownership, which reduces species richness within the Proteobacteria of the infant gut (Tun et al., 2017). From the experimental literature we learn that piglets reared in a natural versus a sterile environment exhibit reduced gut microbial diversity but higher abundance of Enterobacteriales of the gamma-Proteobacteria (Schmidt et al., 2011). Interestingly, Proteobacteria are key microbes linked to natural environmental exposure, skin microbiota diversity, and health in previous studies (Hanski et al., 2012; Ruokolainen et al., 2015). In fact, Proteobacteria are highly present in soil and nature in general, and seem to play a role in immune-related diseases (Shin et al., 2015). In particular, the Proteobacteria, *Acinetobacter lwoffii,* found in cowsheds in rural Europe where rates of atopy are low, has experimentally been found to promote tolerance to allergens and prevent allergic asthma (von Mutius, 2018; Vuitton and Dalphin, 2017). In the recent study by Ruokolainen et al, genus *Acinetobacter* was common on the skin and nares of 6-month old infants in Estonia versus Finland, the former of whom were less likely to develop atopic disease. *E. coli* were also more abundant in the gut microbiota of Estonian infants, who were exclusively breastfed for a shorter time period than Finnish infants but we more likely to live on a farm or close to a forest (Ruokolainen et al., 2020).

Another candidate microbial path for the influence of natural environments may involve the Verrucomicrobiales, dominated by genus *Akkermansia*, which were enriched in our nature-exposed study infants, especially in those exclusively fed formula. Yet, when pets were present, Verrucomicrobiales were reduced in the gut microbiota of formula-fed infants living in close proximity to nature. This pattern coincided with reduced gut microbial diversity and abundance of Clostridiales but enrichment with Enterobacteriales. When genus *Akkermansia* colonize the gut, they dominate (Verster and Borenstein, 2018); however, the activity of mucin-degrading enzymes in *Akkermansia* and hence their survival, can be inhibited by metal ions (Huang et al., 2015). The growth of Proteobacterial, Clostridial and other gut microbial species is also affected by infant exposure to metals (Laue et al., 2020). Trace metals are a common constituent of dust in Canadian homes, especially those occupied by pets, which are credited with tracking soil metals into homes (Rasmussen et al., 2017).

Our study has several strengths. Having access to a novel urban map of natural vegetation and a unique opportunity for data linkage with gut microbial profiles of infants, we were able to evaluate the influence of natural forest, shrub/grass, and wetland environments in a northern climate on infant gut microbiota. Our study population (N = 355) was sizeable for a gut microbiome study and we were able to account for important confounders or mediators, such as levels of urbanization, infant breastfeeding status, and presence of pets at home. However, the size of the study population was limited to test the potential effect of each specific type of natural environment, as the percentage of infants exposed to each of them was relatively low. Similarly, the skewed distribution towards 0 of the exposure variable (with almost 50% of infants not exposed to any natural environment) did not allow estimation of the influence of incremental exposure to a natural environment. On the other hand, while we viewed the variable, household pets, as a vehicle for 'bringing home' or 'increasing the dose' of the natural environment, this variable was limited without additional information on the type of pet and the time it spent outdoors. Finally, given that the main focus of the present study was on natural environments, we excluded maintained green spaces in urban areas, also because these spaces, with their constant anthropogenic disturbance, likely have their own influence on human microbiota (Hui et al., 2017). When the extent of urban green space variable was added to statistical models, nature associations were observed to be in the same direction as in the original model but lost statistical significance, likely due to an over adjustment of the model.

Quite a number of studies have identified the critical role of gut microbiota in infant development and disorders of the immune, metabolic and neurologic systems (Huang et al., 2017; John and Mullin, 2016; Lynch and Pedersen, 2016; Tognini, 2017; Westfall et al., 2017). Scientific evidence also points to the influence of urban green spaces and natural environments on child health (Gascon et al., 2016; Hanski et al., 2012; Ruokolainen, 2017; Tasnim et al., 2017). Ranging from studies which document the influence of design features of green space on bacterial composition of soil (Joyner et al., 2019) to those which find correlations between the amount of Proteobacteria on skin and the amount of residential forest (Ruokolainen et al., 2015), evidence is accumulating on the important role of environmental microbes in mediating associations between natural environments and health benefits observed. Randomized-controlled trials will provide confirmatory evidence for the influence of the natural environment on human microbiota (Gascon et al., 2020), but other study designs are requisite to better understand the mechanisms of nature. Particularly, there is a need for the longitudinal study of the evolution of human microbiota at different body sites from pregnancy to early life, its relationship with natural environment exposure and subsequent implications for human health (Hanson and Gluckman, 2015). In this regard, the inclusion of gut microbiome biomarkers of the host response such as gut

immunoglobulin A, might be an innovative aspect as well (Bridgman et al., 2016; Kang et al., 2018; Mantis et al., 2011). While our study has provided valuable information on the role of breastfeeding and pet ownership in the association between exposure to natural environments and gut microbiota in infants, future studies should consider the spatiotemporal role of air pollution and climate (Liu et al., 2019; Vallès and Francino, 2018), and improve exposure assessments to include details on the vegetation and soil type of natural environments, urban green areas and private gardens, and time spent in these areas, and ideally, to include measures of environmental microbiota. Finally, larger studies are also needed to test the impact of natural environments on gut and skin microbiota beyond indicators of microbial diversity and abundance of dominant taxa.

5. Conclusions

This is the first study to provide evidence of an association between exposure to natural environments in the urban context and human gut microbiota diversity and composition in the first months of life. We observed reduced gut microbiota diversity among those infants exposed to any natural environment, and particularly among those who were formula-fed and lived with household pets. Further research is needed to replicate and better interpret these results, as well as to understand their health consequences.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The CHILD Cohort Study was supported by both the Canadian Institutes of Health Research (CIHR) and the Allergy, Genes and Environment (AllerGen) Network of Centres of Excellence. The authors would like to acknowledge that this work could not have been completed without the cooperation of all members, staff and participants of the CHILD Cohort Study. They include research staff, administrative staff, study families and participants, volunteers, lab technicians, statisticians, and clinical staff. We would also like to acknowledge Ye Peng for his analytical contributions. The Canadian Institutes of Health Research Microbiome Initiative Emerging Team Grant (No. 108028) funded the infant gut microbial profiling.

Mireia Gascon received support to conduct this work at the University of Alberta (Edmonton, Canada) from the Ministerio de Educación, Cultura y Deporte (Spanish Government) in the framework of the Programa Estatal de Promoción del Talento y su Empleabilidad en I + D + i, Subprograma Estatal de Movilidad, del Plan Estatal de I + D + I (José Castillejo grant). Meghan Azad holds a Tier 2 Canada Research Chair in the Developmental Origins of Chronic Disease, and is a CIFAR Fellow in the Humans and the Microbiome Program. Stuart Turvey holds a Tier 1 Canada Research Chair in Pediatric Precision Health. We acknowledge support from the Spanish Ministry of Science and Innovation through the "Centro de Excelencia Severo Ochoa 2019-2023" Program (CEX2018-000806-S), and support from the Generalitat de Catalunya through the CERCA Program.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105881.

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