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Early life microbial exposure, child neurocognition and behaviour at 2 years of age: a birth cohort study

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Declarations of interest

The authors declare no competing financial interests

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obtaining and processing the data and biospecimens. The establishment work and infrastructure for the BIS was provided by the Murdoch Children's Research Institute, Deakin University and Barwon Health. Subsequent funding was secured from the National Health and Medical Research Council of Australia (NHMRC) , The Shepherd Foundation, The Jack Brockhoff Foundation, the Scobie Trust, the Shane O'Brien Memorial Asthma Foundation, the OurWomen's Our Children's Fund Raising Committee Barwon Health, , the Rotary Club of Geelong, the Ilhan Food Allergy Foundation, GMHBA and the Percy Baxter Charitable Trust, Perpetual Trustees. In-kind support was provided by the Cotton On Foundation and CreativeForce. Research at Murdoch Children's Research Institute is supported by the Victorian Government's Operational Infrastructure Support Program. C. Symeonides is supported by a NHMRC PhD scholarship. A.L. Heffernan, J. F. Mueller, A.L. Ponsonby and P. Sly receive NHMRC fellowship support. ES is supported by Australian Rotary Health (Terry Orr Memorial Scholarship), Vanguard and Murdoch Children's Research Institute.

Abstract: Aim: To investigate the relationship between factors influencing external environmental microbial exposures (FEMEs), previously identified to be protective or to increase the risk of the development of allergic disease, and cognition and behaviour in infants 2 years of age in an Australian population.

Method: The Barwon Infant Study is a birth cohort (n=1074) in Victoria, Australia. Comprehensive questionnaire, clinical and biological measures were collected at multiple time-points. Multiple linear regression was used to evaluate associations between 56 FEMEs and three outcomes; cognition (Bayley Scales of Infant and Toddler Development (BAYLEY-III)) (n=667, mean (SD) age = 2.45 (0.14) years), internalising and externalising behaviour (Child Behavior Checklist (CBCL)) (n=666, mean(SD) age = 2.45 (0.14)years).

Results: Overall, there were no consistent patterns or dose response found within an outcome nor across all three outcomes, although there was some evidence for individual associations. Breastfeeding and child care were associated with higher cognitive scores (adj. mean diff. [95%CI] = 3.20 [0.23, 6.17] and 0.68 [0.12, 1.24] respectively), and increasing sibling number was associated with lower internalising behaviour (adj. mean diff. [95%CI] = -4.13 [-6.34, -1.91]).

Conclusion: In contrast to allergic disease, there was an absence of epidemiological evidence to support the association between these FEMEs and cognition and behaviour. Direct investigations into the relationship between exposures which influence gut-microbial composition and cognition and behaviour are now needed.

Key words: microbiome; cognition; Bayley-III; internalising behaviour; externalising behaviour; Child Behaviour Checklist

Brief points**What is already known on this topic**

- Factors which influence external microbial exposures (FEMEs) such as pet ownership, antibiotic use and caesarean section have been identified as important factors in the development of allergic disease.
- Several of these factors impact the infant gut microbial composition.
- The gut microbiome also influences brain development and behaviour, however, the relationship between these factors and cognition and behaviour has not been well characterised.

What this paper adds

- This is the first epidemiological study to investigate relationship between a comprehensive range of both pre- and postnatal FEMEs and cognition and behaviour.

Introduction

There is intense interest in the role of gut microbiome and early cognition and behaviour. Epidemiological studies have identified a range of early-life factors which influence external microbial exposures (termed FEMEs) that are associated with decreased allergic disease risk, some of which also influence the gut microbial composition. Yet little epidemiological work has been conducted on identifying if these factors also relate to infant cognition and behaviour.

The 'hygiene hypothesis' [1] emerged from the observation that larger household size was associated with decreased allergic disease. Subsequent work has shown that multiple factors which increase external microbial exposure are associated with reduced allergic disease, such as; antenatal exposure to farming environments [2] and antenatal pet ownership. Whereas, factors which reduce microbial exposure have been associated with increased allergic disease such as prenatal antibiotic use[3] and caesarean delivery [4].

There is evidence that some of these FEMEs also influence infant gut microbial composition, at least for; mode of delivery [5], antibiotic exposure [5] and pet ownership [6]. Gut microbes are thought to play a crucial role in allergic disease via immune programming and inflammatory mechanisms [7], although, the precise mechanisms remain unclear.

Evidence from germ-free mouse models suggests that the gut microbiome also modulates brain structure, function and behaviour via the microbiota-gut-brain axis [8]. Bacteria involved in the microbiota-gut-brain-axis are hypothesised to perform a more complex role than immune programming and inflammation alone and are thought to be also integral in metabolic, trophic, gene expression modification, protective and structural functions [8].

However, in contrast to allergic disease, there has been minimal investigation of the relationship between FEMEs and cognition and behaviour in humans. Findings from studies

on the allergic disease cannot be extrapolated to cognition and behaviour as the bacteria are involved in different functions. To date, there has been no comprehensive investigation into the relationship between early-life FEMEs, particularly those identified as determinants of the gut microbiome, and cognition and behaviour in humans, although several studies into single candidate FEMEs have been undertaken, predominately investigating the role of mode of delivery on neurodevelopment [9].

Here we undertake an extensive investigation of over 50 FEMEs across the antenatal and postnatal period and their association with infant cognition and internalising and externalising behaviour at 2 years of age. These factors were selected based on past work indicating variation in allergic disease risk. Some factors have been shown to also impact on the gut microbiome. By using this systematic approach, we aim to; investigate a particular factor's influence on an outcome, identify any patterns across the three outcomes, identify any patterns relating to time of exposure and to be able to compare and contrast these findings with those from allergic disease.

Materials and Methods

The Cohort

The Barwon Infant Study (BIS) is an unselected antenatal sampling frame birth cohort study (n=1,074) with antenatal recruitment south-west of Melbourne, Victoria [10].

The Cohort sample

Women were recruited by 28 weeks gestation between June 2010 and June 2013 using an unselected sampling frame. Population characteristics and exclusion criteria have been previously described [10]. This report is based on infants who completed the BAYLEY-III or Child Behaviour Check List (CBCL) at 2 years of age (Figure 1). Multiple births were excluded.

Measures

Exposures

Comprehensive questionnaire data (including sociodemographic profile, life environment, perinatal associated measures and child care attendance) and an extensive range of clinical measures were collected antenatally and at birth, 4 weeks, 3, 6, 9, 12 and 18 months and 2 years [10]. When the same variable was collected at different time points, each time point was considered a separate factor (e.g. maternal antibiotic use in trimesters 1, 2 and 3 was considered as 3 separate factors).

Outcome measures

Cognitive measure

Bayley Scales of Infant and Toddler Development 3rd edition (BAYLEY-III)

The BAYLEY-III[11] cognitive domain was used to measure cognition. The BAYLEY-III was administered at the 2-year review and scored by trained personnel.

Age-standardised cognitive composite scores have a mean score of 100 (SD = 15) [11]. The standardised sample included children with developmental delay (approximately 10%), which had the effect of lowering the overall mean score. Indeed, healthy term Australian children were found have a significantly higher mean score [12]. As we similarly observed a residual age at assessment for the composite scores, we adjusted for the raw scores for post-conceptual age, the age parameter most related to cognition in this cohort. Note in doing this, we are unable to compare the cognitive ability of our cohort with other age-matched cohorts as they used the cognitive composite scores. This was determined to be unimportant as the objective of this study was to investigate the linear relationship between factors influencing FEMEs and cognition and not the cognitive ability of our cohort. The maximum obtainable score is 91.

Behavioural measures

Child Behaviour Checklist (CBCL)

The CBCL /1.5-5[13] internalising and externalising problem scales were used to measure behaviour[13]. T-scores were used in our analysis and have a mean of 50 (SD = 10). Higher scores indicate greater problems. T-scores $60 \geq 63$ represent borderline scores and T-scores > 63 fall into the clinical range. The CBCL questionnaire was collected at the 2-year interview in conjunction with the BAYLEY-III assessment. The CBCL was completed by the infant's carer.

Statistical analysis

Infant and family characteristics are presented as mean (SD) or percentages for those who completed either the BAYLEY-III or the CBCL or both (Table 1). T-tests were used to determine mean differences of continuous variables between the inception cohort and BAYLEY-III/CBCL, while Fisher exact test was used to determine differences in proportions of the categorical variables. Non-parametric tests were used where appropriate. Multiple linear regression was used to evaluate associations between the FEMEs and each outcome (cognition, internalising and externalising behaviour) (Table 2; Note that null associations are recorded at the bottom of the table). Significance is reported at the 5% level. Levels of all external microbial exposures (for example, disinfectant use) were ordered so that the reference group was the level that had the least microbial exposure (e.g. *'everyday'*) to most microbial exposure (e.g. *'don't use'*) (Table 2). Ordering in this manner was undertaken to investigate possible dose response effects, similar to those undertaken in studies in allergic disease. No adjustments for multiple comparisons were undertaken to reduce the risk of type II errors [14]. Stata 15.0 software (StataCorp, College Station, TX) was used for all analyses.

Confounder selection

As the purpose of this study was to screen for preliminary associations only, fully adjusted models are beyond the scope of this paper. All analyses were adjusted for post-

conceptual age and child sex. The cognitive analyses were also adjusted for the processing factors, researcher and researcher experience, which were shown to independently influence the cognitive measure in our cohort.

Ethics

BIS was approved by the Barwon Health Ethics Committee (BHEC 10/24) with informed, written consent obtained from the parents or guardians.

Results

Study populations

The majority of infants in both cohorts were full-term, vaginally-born, and born to Australian-born parents. The mean (SD) age at interview for both the cognitive and CBCL cohort was 2.46 (0.14) years (Table 1). Mothers of infants with BAYLEY-III and CBCL data were marginally older, more likely to have a degree-level education and smoked less than those in the inception cohort. Infants with BAYLEY-III data were breast-fed longer and received more antibiotics than the inception cohort. Less behavioural symptoms were reported on the CBCL than expected from CBCL normative data (Table 1).

Results

Although there were some individual associations (reported below), no consistent patterns of associations or dose response were found within an outcome nor across the three outcomes. Furthermore, no time period appeared more important than any other.

Antenatal period

Maternal antibiotic use in trimester 1, but not trimester 2 or 3, was associated with reduced externalising behaviour (Table 2).

There was an inverse association with the frequency of disinfectant use in T1 and T3 and cognitive scores (Table 2).

Having any older siblings at birth was inversely associated with cognitive scores at 2 years, but positively associated with lower internalising behaviour (Table 2).

Birth

Exposure to labour expressed was associated with higher internalising behaviour compared to infants born to mothers who underwent scheduled C-section (Table 2).

Postnatal Period

Infants who received any breastmilk had higher cognitive scores than infants who received no breastmilk and the longer the infant breastfed, the higher the cognitive scores (Table 2).

Postnatal antibiotic use only between 3 to 6 months was associated with reduced internalising behaviour (Table 2).

Reduced infant hand washing frequency was associated with reduced internalising behaviour, however, evidence was weak (Table 2). A reversal of this relationship was seen at 12m, with reduced infant hand washing frequency associated with increased internalising behaviour (Table 2).

Reduced infant bathing frequency at 6 months was associated with lower cognitive scores and higher externalising behaviour when compared to those infants who were bathed once or more times per day (Table 2).

Dummy use at 4 weeks, 6 or 12 months was associated with lower cognitive scores (Table 2). Length of dummy use (hours) at 4 weeks was also associated with lower cognitive scores. Using the mother's mouth to clean the dummy was associated with higher internalising scores at 4 weeks and 6 months (Table 2).

Childcare attendance was associated with higher cognitive performance than children who did not receive any childcare before the age of 1, although the effect size was small (Table 2).

Hygiene scores

We examined composite hygiene patterns based on the ALSPAC score [15] at 4 weeks, 6 and 12 months, but clear patterns were not detected.

Discussion

Factors altering early life external microbial exposure have been shown to be important in allergic disease programming. The intention of this study was to investigate whether environmental factors previously associated with allergic diseases were also associated with cognition and behavioural outcomes at 2 years of age. Factors also previously linked to gut microbiome were of key interest. In particular, we focused on investigating the strength of a particular factor's influence on individual outcomes, identifying any patterns across the three outcomes, identifying any patterns relating to time of exposure and comparing and contrasting these findings with those from allergic disease.

A factor's influence on an outcome

As expected, breastfeeding was the strongest predictor for cognition, followed by disinfectant use in T3, siblings and weak associations for dummy use bathing frequency and child care attendance. Antenatal disinfectant use at different time periods had the strongest association with both internalising and externalising behaviour. Whereas expected factors, such as siblings and antibiotic use had less influence. No dose response was found with any factor.

Patterns across the three outcomes

In Table 2, although there was perhaps slightly more evidence of associations than expected by chance (20% with $p < \text{ or } = 0.05$ compared to the expected 5%), there were no clear and consistent patterns of association between factors associated with FEMEs and any

outcome. Further, only the association between the number of siblings and the two outcome cognition and internalising behaviour, would have survived Bonferroni correction.

Time of exposure

Multiple time points for multiple variables were investigated. No time point seemed more significant than any other.

Compare and contrast these findings with those from allergic disease.

Unlike studies from asthma and allergy we did not observe an overall pattern that a more sterile environment lead to more adverse outcomes. Indeed, the direction of some associations were opposite to that expected if a more sterile environment is associated with adverse cognitive and behavioural outcomes, such as vaginal delivery and higher internalising behaviour.

Additionally, there were a number of important null findings. Despite dog ownership being associated with reduce allergic disease in both our[16] and a nearby[17] cohorts, dog ownership did not relate to cognition or behaviour. Also, no clear associations were evident with livestock ownership and C-section. Moreover, no consistent associations were found with maternal antibiotic use. Notably, several of these factors have been linked to the gut microbiome.

In contrast to the educative role bacteria play in the immune system where exposure to a greater range of microbes increases the ability of the immune system to be able to distinguish who is friend and who is foe[18], the role bacteria play in the microbiota-gut-brain axis is more diverse and complex [8]. Different patterns of association have been observed in both animal and human studies. Higher alpha diversity has been associated with lower overall cognitive scores in human 2 year-olds [19]. Additionally, mice who have been

treated with antibiotics display reduced microbial diversity, reduced anxiety and increased exploratory behaviour in open spaces [20]. It is likely relationships between FEMEs and cognition and behaviour are non-monotonic. As brain development is multifactorial and intertwined with social influences, future studies teasing out microbial influences from social influences are warranted.

Despite the lack of association between FEMEs and cognition and behavioural outcomes found in this study, the gut microbiome may still play a significant role in cognition and behaviour[21]. Firstly, FEMEs are likely to only explain a small portion of the microbiome variation. Secondly, we only studied FEMEs previously shown to alter allergic disease risk. It may be that other environmental and lifestyle factors that are known to influence brain function and structure and microbiome composition, such as exercise [22], stress [23] or diet [24] are more important for cognition and behaviour than those found to be important for immune programming.

This study's strength is that it is the first epidemiological study to investigate associations between a comprehensive range of early-life FEMEs and three outcomes: cognition, internalising and externalising behaviour, from a population-derived cohort.

There are several limitations to this study. Firstly, as our cohort is an unselected sample, it may not be representative of all mothers and live births. Secondly, as the purpose of this study was to screen for preliminary associations only, models were not fully adjusted and multiple corrections were not applied in order to consider each association on its own [14]. Additionally, many of the reference groups have low numbers. Exposures were analysed in this manner, instead of using a reference group with the highest numbers in order to

investigate a dose-response effect and for consistency with past allergy papers. Furthermore, we recognise that the relationships may be non-monotonic, however, non-monotonic investigations are beyond the scope of this paper. Lastly, our cohort reported less behavioural symptoms than expected based on normative data[13]. There may not be sufficient variation in either the levels of exposure or in the levels of cognition or behaviour in our cohort, limiting our ability to detect associations. Further work would ideally assess trajectories rather than outcomes at a single time point.

Conclusion

Overall there was no consistent pattern across the FEMEs and cognition and behaviour at 2 years of age. In contrast to allergic disease, there was no evidence that markers associated with a more sterile early environment increase neurodevelopmental risk. Direct investigations into gut microbiome compositional differences and cognition and behaviour in humans are now necessary to determine the role the gut microbiome plays in brain function and development.

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Figures and Tables

Figure 1: Flow chart of the participating mother-infant dyads in the initial cohort, BIII and CBCL sample (adapted from [10]).

Table 1: Characteristics of the participants who completed the measure(s).

Infant and family characteristics are presented as mean (SD) or n (percentages) for those who completed either the BAYLEY-III (n=667) or the CBCL (n=666).

Table 2: The relationship between the external microbial exposures and cognition and behaviour

Early life microbial exposure, child neurocognition and behaviour at 2 years of age: a birth cohort study

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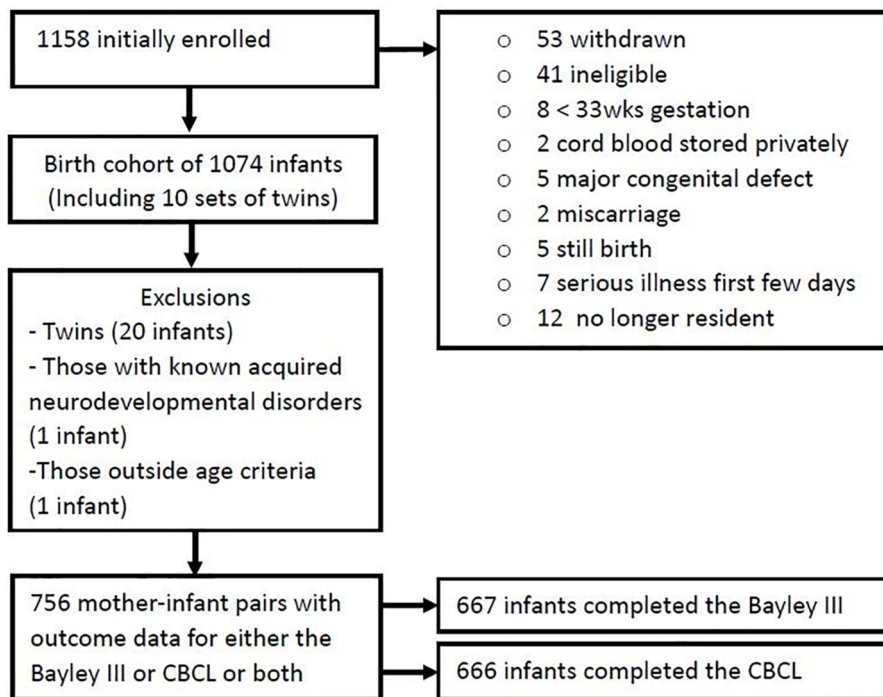
Declarations of interest

The authors declare no competing financial interests

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Table 1: Comparison between the characteristics of the inception cohort with those participants who completed the cognitive and behavioural measures

Characteristics	Inception (n=1074)	Bayley-III (n=667)	CBCL (n=666)
<i>Parent and household</i>			
Maternal age at conception (years)(mean(SD))	31.33(4.79)	31.95(4.57)	32.04(4.36)
Paternal age at conception (years)(mean(SD))	33.49(5.85)	33.73(5.62)	33.97(5.42)
Maternal education (uni degree or above)	548(51.31%)	391(58.89%)	401(60.39%)
Paternal education (uni degree or above)	367(35.15%)	253(38.98%)	260(40%)
Paternal full-time employment	945(91.75%)	586(91.14%)	588(91.45%)
Socioeconomic index for area (SEIFA)			
low	357(33.65%)	200(30.35%)	192(29.14%)
middle	353(33.27%)	226(34.29%)	234(35.51%)
high	351(33.08%)	233(35.36%)	233(35.36%)
Mother born in Australia	967(90.21%)	599(89.81%)	600(90.09%)
Both parents born in Australia	852(82.08%)	527(81.58%)	531(81.94%)
Single parental status in pregnancy	43(4.01%)	21(3.15%)	18(2.7%)
Number of older siblings in the same house at T1T2			
None	483(45.01%)	303(45.5%)	297(44.66%)
One	374(34.86%)	239(35.89%)	239(35.94%)
Two	177(16.5%)	102(15.32%)	111(16.69%)
Three	34(3.17%)	18(2.7%)	16(2.41%)
Four	3(0.28%)	2(0.3%)	1(0.15%)
Five	1(0.09%)	1(0.15%)	0
Six	1(0.09%)	1(0.15%)	1(0.15%)
English - main language at home at 12m	863(97.96%)	632(98.6%)	635(98.76%)
<i>Pregnancy</i>			
Maternal smoking at preconception or in T1	166(15.65%)	75(11.33%)	75(11.31%)
Any maternal smoking in T2 or T3	68(6.4%)	24(3.61%)	25(3.77%)
Any maternal antibiotic use in pregnancy	180(16.84%)	116(17.42%)	111(16.69%)
Maternal pet ownership	788(73.78%)	488(73.38%)	473(71.23%)
Maternal dog ownership	595(68%)	368(67.52%)	361(65.4%)
Maternal livestock ownership	73(6.9%)	50(7.59%)	46(6.98%)
GBS status	212(21.03%)	133(21.01%)	136(21.48%)
<i>Birth</i>			
Gestation age (completed weeks) (mean(SD))	39.44(1.52)	39.49(1.46)	39.49(1.45)
Prematurity			
Pre-term <34	6(0.56%)	4(0.6%)	4(0.6%)
Late pre-term ≥34 < 37	41(3.82%)	21(3.15%)	22(3.3%)
Term ≥ 37 < 42	1027(95.62%)	642(96.25%)	640(96.1%)
Mode of birth			
Unassisted vaginal birth	525(48.88%)	320(47.98%)	318(47.75%)
Forceps	91(8.47%)	58(8.7%)	58(8.71%)
Vacuum	123(11.45%)	84(12.59%)	83(12.46%)

Emergency Caesarean Section	179(16.67%)	104(15.59%)	111(16.67%)
Elective Caesarean Section	156(14.53%)	101(15.14%)	96(14.41%)
Birthweight (kg)(mean (SD))	3.53(0.52)	3.56(0.5)	3.58(0.49)
Birthweight			
low birthweight < 2500g	32(2.99%)	13(1.95%)	11(1.65%)
typical birthweight ≥2500 < 4200g	946(88.33%)	598(89.66%)	601(90.24%)
high birthweight ≥ 4200g	93(8.68%)	56(8.4%)	54(8.11%)
Apgar at 5 min			
< 7	25(2.38%)	13(2%)	15(2.31%)
≥ 7	1024(97.62%)	638(98%)	635(97.69%)
Child			
Infant sex (female)	519(48.32%)	306(45.88%)	313(47%)
Breastfeeding (weeks)(mean(SD))	31.49(20.02)	34.13(19.23)	34.3(19.07)
Duration of breastfeeding in the first 12m			
None	22(2.28%)	6(0.91%)	10(1.52%)
Any breastmilk feeding > 0 < 1wk	113(11.7%)	58(8.77%)	58(8.81%)
Any breastmilk feeding ≥ 1wk < 6m	255(26.4%)	168(25.42%)	158(24.01%)
Any breastmilk feeding ≥ 6m < 12m	210(21.74%)	145(21.94%)	152(23.1%)
Any breastmilk feeding ≥ 12m	366(37.89%)	284(42.97%)	280(42.55%)
Postnatal antibiotic use in the first 12m	439(40.88%)	308(46.18%)	302(45.35%)
Attended day care centre in the first 12m	412(41.04%)	304(45.65%)	298(44.81%)
Bayley-III raw score (mean(SD))	71.14(4.12)	71.18(3.95)	71.35(3.92)
Bayley-III scaled score (mean(SD))	10.76(2.08)	10.79(2.04)	10.9(2.02)
Internalising behaviour(mean(SD))	41.58(9.19)	41.78(9.09)	41.68(9.14)
Externalising behaviour(mean(SD))	44.90(9.26)	44.83(9.02)	44.82(9.20)
Age at 2-year interview (years) (mean(SD))	2.45(0.14)	2.45(0.14)	2.45(0.14)

Note: bolded type indicates a $p < 0.05$ difference between the inception cohort and the individual cohorts

Table 2: The relationship between factors influencing external microbial exposure and cognition and behaviour

Factors influencing External Microbial Exposures (EMEs)	Bayley III(cognition)		Internalising CBCL		Externalising CBCL	
	Adj mean diff ^a [95%CI]	P-value	Adj mean diff ^b [95%CI]	P-value	Adj mean diff ^b [95%CI]	P-value
<i>Antenatal period</i>						
Livestock ownership ^c	0.07 [-1.00, 1.15]	0.89	0.06 [-2.98, 3.11]	0.97	-1.14 [-4.18, 1.90]	0.46
Any farm work	0.60 [-0.24, 1.44]	0.16	-0.75 [-3.19, 1.69]	0.55	0.63 [-1.81, 3.08]	0.61
Any pets ^d	-0.39 [-1.03, 0.25]	0.23	0.27 [-1.47, 2.00]	0.76	-0.96 [-2.69, 0.76]	0.27
Maternal pet dog	-0.31 [-0.98, 0.36]	0.36	0.29 [-1.52, 2.10]	0.75	-1.20 [-2.99, 0.59]	0.19
Any fever in T1 ^e	0.12 [-1.15, 1.40]	0.85	-0.03 [-3.85, 3.80]	0.99	-3.38 [-7.13, 0.38]	0.08
Antibiotic use in T1 ^f	0.45 [-1.03, 1.93]	0.55	-1.94 [-5.93, 2.05]	0.34	-4.42 [-8.32, -0.52]	0.03
Disinfectant use in T1						
Every day	Reference		Reference		Reference	
A few times/wk	-0.56 [-2.12, 1.00]	0.48	0.20 [-5.08, 5.49]	0.94	-2.53 [-7.80, 2.74]	0.35
About 1/wk	0.62 [-0.77, 2.02]	0.38	-2.64 [-7.12, 1.83]	0.25	-5.92 [-10.39, -1.46]	<0.01
< 1/wk	0.52 [-0.84, 1.88]	0.46	-1.85 [-6.23, 2.54]	0.41	-4.26 [-8.64, 0.11]	0.06
Don't use	0.87 [-0.46, 2.21]	0.20	-0.61 [-4.95, 3.72]	0.78	-3.86 [-8.19, 0.46]	0.08
<i>Trend</i>		0.03		0.88		0.44

Factors relating to External Microbial Exposures (EMEs)	Bayley III(cognition)		Internalising CBCL		Externalising CBCL	
	Adj mean diff ^b [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value
Disinfectant use in T2						
Every day	Reference		Reference		Reference	
A few times/wk	-0.02 [-1.56, 1.53]	0.98	0.73 [-4.38, 5.84]	0.78	-1.53 [-6.68, 3.61]	0.56
About 1/wk	0.82 [-0.58, 2.22]	0.25	-3.14 [-7.54, 1.27]	0.16	-6.92 [-11.35, -2.48]	<0.01
< 1/wk	0.78 [-0.58, 2.15]	0.26	-2.64 [-6.94, 1.66]	0.23	-5.26 [-9.59, -0.93]	0.02
Don't use	0.89 [-0.45, 2.22]	0.19	0.22 [-4.01, 4.45]	0.92	-3.70 [-7.96, 0.57]	0.09
<i>Trend</i>		<i>0.09</i>		<i>0.50</i>		<i>0.45</i>
Disinfectant use in T3						
Every day	Reference		Reference		Reference	
A few times/wk	0.99 [-0.86, 2.84]	0.29	7.43 [0.67, 14.19]	0.03	5.84 [-0.89, 12.58]	0.09
About 1/wk	2.51 [0.73, 4.28]	<0.01	5.32 [-1.18, 11.83]	0.11	4.20 [-2.27, 10.68]	0.20
< 1/wk	2.44 [0.72, 4.17]	<0.01	6.10 [-0.26, 12.46]	0.06	5.06 [-1.27, 11.39]	0.12
Don't use	2.34 [0.56, 4.12]	<0.01	5.40 [-1.08, 11.89]	0.10	3.52 [-2.94, 9.98]	0.28
<i>Trend</i>		<0.01		<i>0.96</i>		<i>0.73</i>

Factors relating to External Microbial Exposures (EMEs)	Bayley III(cognition)		Internalising CBCL		Externalising CBCL	
	Adj mean diff ^b [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value
Total no. of adults at home (T1T2)						
One	Reference		Reference		Reference	
Two	0.84 [-1.33, 3.01]	0.45	-2.74 [-8.18, 2.71]	0.32	-2.39 [-7.9, 3.11]	0.39
Three	1.42 [-1.23, 4.06]	0.29	2 [-4.61, 8.61]	0.55	-2.73 [-9.41, 3.95]	0.42
Four or more	-1.46 [-4.5, 1.58]	0.35	-5.97 [-13.79, 1.84]	0.13	-2.78 [-10.69, 5.12]	0.49
<i>Trend</i>		<i>0.42</i>		<i>0.98</i>		<i>0.80</i>
Total no. of children at home (T1T2)						
None	Reference		Reference		Reference	
One	-0.34 [-0.97, 0.29]	0.28	-3.75 [-5.47, -2.02]	<0.001	0.16 [-1.58, 1.90]	0.86
Two	-1.45 [-2.28, -0.62]	<0.001	-4.13 [-6.34, -1.91]	<0.001	0.60 [-1.64, 2.84]	0.60
Three	-1.58 [-3.25, 0.08]	0.06	-3.05 [-7.85, 1.76]	0.21	2.65 [-2.22, 7.51]	0.29
Five or more	-2.36 [-5.11, 0.40]	0.09	-3.88 [-13.02, 5.27]	0.41	2.46 [-6.80, 11.71]	0.60
<i>Trend</i>		<0.001		<0.001	<i>0.31</i>	

Factors relating to External Microbial Exposures (EMEs)	Bayley III(cognition)		Internalising CBCL		Externalising CBCL	
	Adj mean diff ^b [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value
<i>Birth</i>						
Mode of birth						
Scheduled C-section	Reference		Reference		Reference	
Unscheduled C-section	0.48 [0.54, 1.50]	0.35	3.67 [1.18, 6.15]	0.04	1.31 [-1.21, 3.83]	0.31
Instrumental vaginal delivery [§]	-0.03 [-0.97, 0.90]	0.94	3.76 [1.50, 6.02]	<0.001	1.56 [-0.73, 3.85]	0.18
Unassisted vaginal delivery	0.17 [-0.65, 0.99]	0.68	2.17 [0.20, 4.13]	0.03	1.36 [-0.63, 3.35]	0.18
Group B Streptococcus (GBS)	-0.27 [-0.98, 0.44]	0.45	1.81 [-.013, 3.75]	0.07	1.34 [-.60, 3.29]	0.18
<i>Postnatal period</i>						
Any Breastfeeding	3.20 [0.23, 6.17]	0.04	1.37 [-4.49, 7.23]	0.65	1.28 [-4.53, 7.09]	0.67
Breastfeeding duration						
0 < 1wk	Reference		Reference		Reference	
≥1 wk < 6m	0.20 [-1.49, 1.88]	0.82	-2.56 [-6.74, 1.62]	0.23	-1.83 [-5.99, 2.33]	0.39
≥6m	1.05 [-0.60, 2.70]	0.21	-1.47 [-5.50, 2.56]	0.48	-1.86 [-5.87, 2.15]	0.36
<i>Trend</i>		0.005		0.64		0.56

Factors relating to External Microbial Exposures (EMEs)	Bayley III(cognition)		Internalising CBCL		Externalising CBCL	
	Adj mean diff ^b [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value
Infant pet dog	-0.16 [-0.75, 0.42]	0.59	-0.36 [-1.95, 1.23]	0.66	-1.57 [-3.16, 0.02]	0.05
Any antibiotic use between 3m-6m ^h	0.41 [-0.52, 1.35]	0.39	-3.06 [-5.55, -0.56]	0.02	-0.53 [-3.02, 1.96]	0.67
Total antibiotic days to 12m ⁱ	0.02 [-0.01, 0.05]	0.27	-0.01 [-0.09, 0.07]	0.82	0.04 [-0.04, 0.12]	0.33
Mat. handwashing method at 4wks ^l						
Wash hands with sanitiser	Reference		Reference		Reference	
Wash hands with soap	0.22 [-0.39, 0.83]	0.48	0.37 [-1.32, 2.06]	0.67	0.26 [-1.42, 1.94]	0.76
Wipe hands only	1.68 [-0.15, 3.51]	0.07	-1.52 [-6.99, 3.95]	0.59	2.96 [-2.50, 8.42]	0.29
Rinse with water only	-0.55 [-2.03, 0.92]	0.46	-0.31 [-4.73, 4.10]	0.89	-0.46 [-4.87, 3.95]	0.84
Don't clean hands	-0.02 [-3.02, 2.97]	0.99	3.23 [-5.09, 11.54]	0.45	1.61 [-6.69, 9.91]	0.70
Infant handwashing freq. at 4wks ^k						
5 or more times/d	Reference		Reference		Reference	
3-4 times/d	1.43 [-1.10, 3.97]	0.27	-2.98 [-9.59, 3.63]	0.38	-4.57 [-11.15, 2.00]	0.17
1-2 times/d	1.64 [-0.49, 3.77]	0.13	-4.43 [-9.84, 0.98]	0.11	-5.66 [-11.04, -0.28]	0.04
Not at all	1.34 [-0.80, 3.48]	0.22	-5.19 [-10.62, 0.25]	0.06	-5.19 [-10.60, 0.21]	0.06
Trend		0.99		0.05		0.40

Factors relating to External Microbial Exposures (EMEs)	Bayley III(cognition)		Internalising CBCL		Externalising CBCL	
	Adj mean diff ^b [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value
Infant handwashing frequency at 12m						
5 or more times/d	Reference		Reference		Reference	
3-4 times/d	-0.06 [-0.73, 0.60]	0.85	0.07 [-1.70, 1.85]	0.93	-0.46 [-2.25, 1.33]	0.61
1-2 times/d	0.01 [-0.88, 0.89]	0.99	2.99 [0.67, 5.31]	0.01	2.54 [0.20, 4.89]	0.03
Not at all	-2.11 [-7.30, 3.07]	0.42	6.39 [0.18, 12.60]	0.04	-0.29 [-6.56, 5.97]	0.93
<i>Trend</i>		<i>0.86</i>		0.03		<i>0.08</i>
Infant bathing frequency at 6m ^l						
Once or more a day	Reference		Reference		Reference	
Several times a wk	-0.63 [-1.23, -0.04]	0.04	0.07 [-1.53, 1.67]	0.93	1.56 [-0.03, 3.14]	0.05
Once a wk or less	-0.80 [-2.40, 0.81]	0.33	3.30 [-1.39, 7.99]	0.17	5.21 [0.56, 9.85]	0.03
<i>Trend</i>		0.03		<i>0.48</i>		0.01
Dummy use at 4wks	-0.66 [-1.25, -0.08]	0.03	0.44 [-1.16, 2.04]	0.59	0.59 [-1.00, 2.18]	0.46
Length dummy use at 4wks (hrs/day) ^m	-0.14 [-0.26, -0.01]	0.03	-0.10 [-0.49, 0.28]	0.60	-0.16 [-0.55, 0.23]	0.41

Factors relating to External Microbial Exposures (EMEs)	Bayley III(cognition)		Internalising CBCL		Externalising CBCL	
	Adj mean diff ^b [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value	Adj mean diff ^c [95%CI]	P-value
Method of dummy cleaning at 4wks						
Sterilizing agent	Reference		Reference		Reference	
Wash boiling water	0.55 [-0.43, 1.54]	0.27	1.38 [-1.44, 4.21]	0.34	2.02 [-0.81, 4.86]	0.16
Wash tap water	0.53 [-0.67, 1.73]	0.38	1.94 [-1.55, 5.44]	0.27	1.30 [-2.21, 4.80]	0.47
Own mouth	1.08 [-0.22, 2.37]	0.10	3.98 [0.17, 7.79]	0.04	3.27 [-0.56, 7.09]	0.09
W/o washing	1.66 [-0.18, 3.51]	0.08	4.45 [-1.34, 10.24]	0.13	5.04 [-0.77, 10.85]	0.09
Dummy use at 6m	-0.96 [-1.54, -0.38]	>0.01	0.21 [-1.38, 1.80]	0.80	0.41 [-1.17, 1.99]	0.61
Method of dummy cleaning at 6m ⁿ						
Sterilizing agent	Reference		Reference		Reference	
Wash boiling water	-0.29 [-1.78, 1.20]	0.70	1.19 [-2.99, 5.36]	0.58	-1.64 [-6.02, 2.73]	0.46
Wash tap water	0.51 [-0.97, 2.00]	0.50	2.41 [-1.62, 6.45]	0.24	1.75 [-2.48, 5.97]	0.42
Own mouth	1.22 [-0.39, 2.83]	0.14	4.76 [0.23, 9.28]	0.04	3.87 [-0.88, 8.61]	0.11
W/o washing	0.72 [-0.86, 2.30]	0.37	0.98 [-3.44, 5.40]	0.66	0.62 [-4.01, 5.26]	0.79
Dummy use at 12m	-0.60 [-1.18, -0.02]	0.04	-0.86 [-2.45, 0.72]	0.28	-0.60 [-2.20, 0.99]	0.46
Any formal child care to 12m ^o	0.68 [0.12, 1.24]	0.02	-1.17 [-2.74, 0.39]	0.14	-1.26 [-2.82, 0.29]	0.11

^a adjusted for age at BAYLEY-III interview, child sex, researcher and research assistant experience in administering the BAYLEY-III.

^b adjusted for age at interview and child sex.

^c No association was found with chicken, cow, sheep, horse ownership.

^d No association was found with whether the pets were kept inside, outside or both.

^e No association was found with fever in T2 or T3.

^f No association was found with maternal antibiotic use in T2 or T3.

^g Forceps or vacuum.

^h No association was found with any infant antibiotic use to 4 wks, between 4 wks and 3m, 6m to 9m, 9m to 12m.

ⁱ No association was found with total number of antibiotic days to 4 wks, between 4 wks and 3m, 3m to 6m, 6m to 9m, 9m to 12m.

^j No association was found with maternal handwashing method at 6m or 12m.

^k No association was found with infant hand washing frequency at 4wks.

^l No association was found with infant bathing frequency at 4 wks or 12m.

^m No association was found with length of dummy use at 6m or 12m.

ⁿ No association was found with method of dummy cleaning at 12m.

^o No association was found with age infant first attended child care (wks) to 12m.