Sex dependent associations between microbiome disruption in infancy and the prevalence of behavioural disorders at 81 months

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Introduction

Humans evolved from our primate ancestors within complex ecosystems already richly populated with a wide variety of microscopic organisms (Stilling, Dinan, & Cryan, 2014). During prolonged periods of coevolution, humans developed complex relationships with many of the organisms present in their environment. While some of these relationships are primarily parasitic, many are more symbiotic in nature (Wang & Kasper, 2014; Gilbert, 2014). For example, humans provide the commensal bacteria that occupy their bodies with a physical habitat, as well as energy and other resources, while the presence of the bacteria allow humans to extract additional nutrients from their food (Song, Dominguez-Bello & Knight, 2013), help to calibrate and educate the immune system (Round & Mazmanian, 2009), and produce a variety of useful metabolic bi-products (Tillisch, et al. 2012).

The microbiome is a term used to describe the sum of all the genetic material provided by the microscopic organisms that occupy the human body (Wang & Kasper, 2014). The human microbiome can be composed from over 1,000 different bacterial species, but the average western microbiome is composed from approximately 160 (Qin J, et al, 2010). It is estimated that the average microbiome is composed of approximately 40 trillion cells, significantly outnumbering the 30 trillion cells that make up the host organism (Sender, Fuchs & Milo, 2016). The microbiome provides of wealth of genetic material that can greatly expand the capabilities of the host organism, and it viewed by some as an essential organ of the human body (Qin J, et al, 2010; Lloyd-Price, Abu-Ali & Huttenhower, 2016).

During a natural human birth, bacteria immediately begin to colonize the internal and external surfaces of the infant's body as it travels through the birth canal (Dominguez-Bello, et al. 2010). The microbial populations in a women's vagina begin to shift as birth approaches,

assumed to be in preparation for this initial inoculation (Dominguez-Bello, et al. 2010). During the birthing process the infant will primarily be exposed to microbes from the mother's skin, vagina, and anus, as well as other microbes present in the environment, such as those present on hospital equipment or hospital staff (Marques, Cryan, Shanahan, Fitzgerald, Ross, Dinan, & Stanton, 2014; Dominguez-Bello, et al., 2010). From the point of initial colonization the child's microbiome will experience rapid shifts in the total volume of bacteria, as well as in proportional composition (Song, Dominguez-Bello, & Knight, 2013). The volume and composition of the microbiome typically begins to stabilize around three years of age, but will continue to change and adapt throughout the lifespan (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014).

Modern medicine and changing lifestyle factors have led to dramatic changes in the ecology of the modern human microbiome, most notably in the intestinal microbiome (Marques, Cryan, Shanahan, Fitzgerald, Ross, Dinan, & Stanton, 2014). The intestinal microbiome refers to the microbes that occupy the gastrointestinal (GI) tract, stomach, small intestine, and colon (Chen, D'Souza & Hong, 2013). Increasingly sterile environments, changes in diet, and the applications of modern medical practices have led to the eradication of specific pathogenic bacteria and parasites, as well as significant decreases in the overall ecological diversity of the microbiome (Blaser, 2014). Medical practices, such as the administration of antibiotics during pregnancy and early life, birth by caesarean section, and a lack of breastfeeding have been shown to have notable and lasting impacts on the human microbiome (Korpela, et al., 2016). Although there is no agreed upon microbiome signature for optimal health, like most ecosystems, a higher diversity is associated with better health outcomes (Lloyd-Price, Abu-Ali, & Huttenhower, 2016).

A growing number of observational studies have demonstrated associations between exposure to antibiotics, lack of breastfeeding, and birth by caesarean with an increased risk of metabolic and immune-related disorders (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014; Mueller, et. al, 2015; Korpela, et. al., 2016; Lloyd-Price, Abu-Ali & Huttenhower, 2016). Although these studies are largely observational in nature, the proposed causal mechanisms are supported by an growing number of experimental studies (Turnbaugh, et. al. 2006; Jarmila, et al., 2016). These experimental studies, largely in rodents, have clearly demonstrated that microbiome disruption can directly impact physiological functions such as metabolic efficiency (Devaraj, Hemarajata, & Versalovic, 2013), as well as impact gut-barrier function and modulate immune responses (Round & Mazmanian, 2009).

An emerging area of research suggests that alterations in the microbiome may also be associated with changes in the central nervous system (CNS), and may ultimately influence the behaviour of the host organism (Wang & Kasper, 2014; Stilling, Dinan, & Cryan, 2014). The proposed mechanism for many of these behavioural effects is the gut-microbiota-brain-axis (Chen, D'Souza, & Hong, 2013). The gut-microbiota-brain-axis refers to a bi-directional communication network between the microbiota in the intestinal microbiome and the host's CNS (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). This communication is facilitated through a variety of mechanisms, including the valgus nerve; production of inflammatory cytokines; and various neuroactive chemicals, including precursors to important neurotransmitters like serotonin (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). Current research on the relationship between the microbiome, various neurological factors and behaviour in humans and other mammals has focused on primarily four main approaches: use of germ-free (GF) mice, antibiotic

exposure, exposure to probiotics or infectious agents, and fecal transplantation (Cryan & Dinan, 2012).

GF experimentation utilizes the sterile uterine environment and specific birthing processes to produce test subjects that are essentially free of bacterial colonization. This allows for direct comparisons of behavioural characteristics between sterile and non-sterile members of the same population. Several studies using GF mice have demonstrated notable changes in behaviour and levels of various neurochemicals using this methodology (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). For example, recent studies have reported that male GF mice have altered neuroendocrine responses to stress, and exhibit reduced anxiety-like behaviour when exposed to novel and stressful environments (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). Similar behavioural changes are then manifested in subsequent generation of mice due to a lack of bacteria inherited during the birthing process (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). Interestingly, the reduced anxiety-like behaviour could be normalized by reintroducing normal bacteria to the GF mice in the post-weaning period (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). The reversal of the behavioural changes did not occur if recolonization was delayed until adulthood, suggesting a specific window of vulnerability in early life that may result in life long changes to the CNS (Foster, & Neufeld, 2013; Cryan, & Dinan, 2012). GF rearing as also been shown to negatively impact the social behaviour of male mice which have been shown to display aversion to normal social interaction when compared to normally reared mice (Desbonnet, Clarke, Shanahan, Dinan and Cryan, 2014).

Previous studies suggest many of the neurological and behavioural effects of GF rearing appears to be more pronounced in male rodents. Male GF mice have been shown to have increased levels of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the

hippocampus, as well as plasma levels of circulating tryptophan (Forsythe, Kunze & Bienenstock, 2012; Clarke, Grenham, Scully, Fitzgerald, Moloney, Shanahan, Cryan, 2013; Clark, et al., 2013), similar effects have not been observed in female GF mice. Altered levels of brain derived neurotrophic factor (BDNF) have also been observed, but again only in male GF mice (Clark, et al., 2013). In a more recent study, male GF mice have been shown to exhibit significant changes in levels of myelination during development of the prefrontal cortex, when compared to normally reared mice; again, this effect was only present in male GF mice (Hoban, et al., 2016).

Taken together these findings suggest there may be important phenotype (sex)-environment (bacteria) interactions influencing the effects of microbiome disruption on CNS development and function, and it appears the effects may be more pronounced in males. Altered levels of 5-HT have long been associated with memory and mood disorders (Autry & Monteggia, 2012). Alterations of BDNF production (Green, Matheson, Shepherd, Weickert & Carr, 2011) and disordered myelination have been associated with the development of schizophrenia (Davis, et al., 2003). This is interesting, as rates of certain neurodevelopmental conditions, such autism, and other behavioural disorders appear to be increasing and disproportionally affect males (Cryan & Dinan, 2012).

Another method of exploring microbiota driven behavioural changes in mammals is through the application of antimicrobial drugs. Similar to the effects observed in GF mice, oral administration of antimicrobials in adult mice resulted in decreased anxiety-like behaviour and altered levels of BDNF in both the hippocampus and amygdala, after controlling for possible off-target effects (Cryan, & Dinan, 2012). These results further support the hypothesis that destabilizing a mammal's microbiome through the application of antimicrobial drugs can alter

levels of various neuroactive agents and result in observable changes in their behaviour. Unfortunately, there is limited data to date directly comparing the impact of antimicrobial drugs across sexes and this is an area that should be further developed based on the results of the GF studies.

The application of probiotics is another common method of studying the impact of the gut microbiota on cognition and behaviour. Probiotics are defined as live bacterial organisms that, when ingested, result in beneficial changes in the health status of the host organism (Cryan, & Dinan, 2012). Previous studies have demonstrated that probiotics can induce measurable effects on the behaviour of rodents and humans (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). The two genera that have been studied most extensively for their health benefits are Bifidobacteria and Lactobacillus (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). Important individual differences in the effects of these two probiotics have been found, indicating that initial health status of the host and other factors play a large role in moderating their effects (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014).

Probiotics have shown to help mitigate the stress response from external stressors, such as maternal separation, social disruption, and restraint stress in rodents (Marques, et. al., 2014). In one such study, rats given a probiotic cocktail exhibited reduced "depression-related behavioural effects" following myocardial infarction (Cryan, & Dinan, 2012). In another recent study, ingestion of Lactobacillus strain resulted in decreased anxiety-like behaviour in mice, as well as decreased responsiveness to stress (Marques, et. al., 2014). These studies demonstrate that positive changes in the microbiome through ingestion of certain commensal bacteria may induce favourable behavioural effects or help to mitigate negative effects of other stressors.

Significant effects on cognition and behaviour have also been observed in probiotic studies using human subjects (Cryan, & Dinan, 2012). Recent clinical trails have demonstrated that probiotics can reduce anxiety, mimic antidepressant-like effects, decrease overall psychological distress, decrease serum cortisol levels, and increase resistance to stress-induced damage to the mucosal lining of the intestines (Cryan, & Dinan, 2012). In a recent landmark study, healthy human female subjects with no reported psychiatric conditions or GI issues were randomized to receive either a live culture fermented milk product, or a non-fermented milk beverage containing no live bacteria, twice daily for a four-week period. Functional magnetic resonance imaging (fMRI) was conducted prior to and four weeks after study initiation to measure both brain response to an "emotional faces attention task" and resting brain activity (Tillisch, et al., 2012). Multivariate and region of interest analyses indicated that intake of the live culture was associated with significant changes in brain activation when exposed to emotional images, when compared to those receiving the placebo. The study suggests that in human female subjects the probiotic resulted in a reduction in attentiveness to negative environmental stimuli. This finding suggests that the probiotic microbiota may alter the processing of sensation and emotion in the female brain in a way which may influence psychological wellbeing (Tillisch, et al., 2012).

While over a decade of research has associated psychiatric side effects with antibiotic exposure (Rogers, Keating, Wong, Licino, & Wesselingh, 2016), but since a potential causal mechanism for these reported effects had yet to be identified, these effects were largely dismissed. Recently, in a large population-based case control study using the United Kingdom (UK) prescription and mental health data reported that a single course of antibiotics resulted in an increased risk of anxiety and depression among patients between the ages of 15 and 65 (Lurie,

Haynes, Mamtani, & Bursi, 2015). The researchers reported a dose-dependent relationship, with the risk increases from subsequent doses of antibiotics (Lurie, Haynes, Mamtani, & Bursi, 2015). The authors summarize that these findings were consistent with previous animal models, indicating dysbiosis may result in an amplified Hypothalamic Pituitary Axis (HPA) stress response and changes in serotonergic and noradrenergic pathway; factors that have been linked to the development of depression and anxiety in humans (Lurie, Haynes, Mamtani, & Bursi, 2015). However, there were important limitations to this exploratory study, including the relatively methodological weakness of case-control designs, and a disregard for potentially important sex-exposure interactions that may have moderated the outcome. Similar to previous observational studies using various physiological outcomes in humans it is difficult to control for potential confounding resulting from the health status which warranted the antibiotic intervention.

A proposed by the developmental origins of health and disease (DOHaD) hypothesis (Barouki, Gluckman, Grandjean, Hanson, & Heindel, 2012), the current literature suggests there may be long-term health consequences resulting from dysbiosis in the postnatal period. Despite widespread use of antibiotics in infancy, and a growing body of evidence suggestion potential risks, no previous studies have been conducted to directly examine the relationship between exposure to antibiotics in infancy and the development of behavioural disorders in children.

The current study aims to explore the relationship between exposure to antibiotics within three separate windows in the first 24 months of life (0-5 months, 6-14 months, and 15-23 months) and the development of behavioural disorders at 81 months of age using a commonly utilized and well validated screener of behavioural disorders in children, the Strength and Difficulties Questionnaire (SDQ).

Previous research suggests there may be a specific window of vulnerability in the 0 to 24-month period (Trasande, Blustein, Liu, Corwin, Cox, & Blaser, 2013), so multiple time points of exposure were explored to determine if earlier exposures are more strongly associated with the outcome. Numerous studies in animal models have also demonstrated clear sex-dependent effects of microbiome disruptions on brain development and function, therefore sex was included as potential effect modifier (Clarke, et al., 2013; Neufeld, 2010). It was hypothesized that earlier periods of exposure will results in larger behavioural effects and the effect is likely to vary significantly based on the sex of the child. It is unclear exactly how the behavioural effects resulting from dysbiosis of the microbiome in infancy will manifest in humans, but the rodent models suggest that the associated neurological and behavioural changes may be more pronounced in males.

Subjects and Methods

Data source and sample

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a longitudinal birth cohort study established in the early 1990's in Avon, UK. Avon is a geographic area 120 miles west of London which encompasses the city of Bristol (population 0.5 million) and surrounding areas including smaller towns, villages and rural communities. Any pregnant women with an expected delivery date between April 1991 and December 1992 were eligible for participation. Approximately 85% of the pregnant mother eligible for the study returned at least one questionnaire, generating a robust sample largely representative of the overall UK population (Fraser, et. al, 2013). The purpose of the study was to identify important factors that impact child development. During the study data were collected from a variety of sources including surveys, medical records, and direct examinations.

At study onset, 14,541 pregnant women were recruited, and of these pregnancies 13,972 infants were alive at the end of year one. Of the infants alive at year one, 9,431 (68%) had antibiotic exposure data for all three of the exposure windows (0-5 months, 6-14 months, and 15-23 months). The variable with the highest degree of missing data was the SDQ total score measured at 81 months (missing in 55.3% of cases alive at the end of year one). A complete cases analysis using all exposures outcomes and covariates resulted in an analytic sample of 3,468 (25% of the total infants alive at the end of year one). See appendix B for a full description of the proportion of missing data for all variables included in the study. Although the drop-out rate and resulting proportions of missing data were considerable, they are consistent with comparable large-scale longitudinal studies with multiple waves of data collection over the life span. (Wolke, et al., 2009). The potential impacts and biases introduced are discussed in more detail later.

Measures

Exposures to antibiotic

The ALSPAC used a series of self-report mail questionnaires to determine if the children had been exposed to antibiotics in the specified time window. Measurements were taken at the end of each time interval, reflecting on the previous 6-9 months period. Exposures at 0-5 months of age were obtained at 6 months, exposures between 6–14 months were obtained at 15 months, and exposures between 15-23 months were obtained at 24 months. The questionnaire at the 6-month mark stated: ‘Children often have accidents or illnesses that need treatment. Please indicate whether antibiotics have been given to your child in the past 6 months’. The questions posed at each of the other time intervals were identical, apart from the time window referenced. The response options allowed parents to indicate ‘none’, ‘one’ or ‘more than one’ exposure. Parents were also asked the type of antibiotic the child was exposed to, but this data was found to

be unreliable in previous studies and was discarded (Trasande, Blustein, Liu, Corwin, Cox, & Blaser, 2013). Parents were not asked the medical reason why the antibiotics were prescribed, a potential limitation that will be discussed later. Based on the parent's self -report the subjects were classified as either exposed or unexposed, to one or more antibiotics in the specified time window. Those exposed were coded as "1" and those unexposed were coded as "0".

Behavioural outcomes

Behavioural problems were assessed through the mother's self-report on the SDQ (Goodman 1997) when the child was approximately 81 months of age. The SDQ is a very well established and validated psychological screener used internationally for epidemiological research and mental health screening at the population level and within clinical samples (Warnick, Bracken, & Kasl, 2008). The SDQ is comprised of 25 items, broken down in five sub-scales: hyperactivity, emotional symptoms, conduct problems, peer problems, and pro-social behaviour. Each subscale produces a minimum score of 0 and a maximum score of 10. Four out of five of the subscales are combined to create a total score ranging from 0 to 40. The fifth subscale, which looks at pro-social behaviours, is not included in the total score. Similar to previous studies using the SDQ, subscale scores in the current dataset were pro-rated if less than three items were missing (Peacock, Lewis, Northstone, & Wiles, 2011). A higher score indicates a higher degree of emotional and behavioural problems on all subscale except for pro-social behaviour, where a lower score indicates greater difficulties.

A recent systematic review concluded the SDQ possessed adequate reliability and validity as a measure of psychopathology when administered to parents and caregivers in large population-based samples (Warnick, Bracken, & Kasl, 2008). A recent paper looking at the psychometric properties of the SDQ reported an internal consistency ranging from good (a =.77) to excellent (a=.90) for the total score based on a parent's self report (Stone, Janssens, Vermulst,

Van Der Maten, Engels, & Otten, 2015). The SDQ total score has also been shown to be a valid measure of a mental health severity and a significant predictor mental health service utilization (Warnick, Bracken, & Kasl, 2008).

The SDQ total score was selected *a priori* as the outcome for the current study because of its higher reliability, predictive validity in relation to mental health service utilization, and its ability to detect changes in a broader range of behavioural characteristics, when compared to the individual subscales (Warnick, Bracken, & Kasl, 2008; Bourdon, Goodman, Rae, Simpson, & Koretz, 2005).

Due to a strong positive skew in the SDQ data, which could not be corrected by transformation, participants scores were classified as either normal/borderline or abnormal using the child's sex and age-specific abnormal cut-offs identified in a recent multi-cohort population-based sample (Niclasen, Teasdale, Andersen, Skovgaard, Elberling, & Obel, 2012). See appendix A for full data on the attempt at normalizing the SDQ total score. The abnormal cut-offs used in the current study are provided in Table 1.

Table 1: SDQ cut offs for Danish 5-7-year-olds, parent ratings

	Recommended score bandings for boys (n = 28,920)			Recommended score bandings for girls (n = 27,611)		
	Normal	Borderline	Abnormal	Normal	Borderline	Abnormal
Total Difficulties	0-9	10-13	14-40	0-8	9-11	12-40

Potential confounders

A set of social, behavioral and biological pre-natal or early-life predictors of childhood behavioural problems were included as covariates to control for potential confounding. These included maternal age at birth, maternal education (defined using UK Office of Qualifications and Examinations Regulation level), social class (defined using UK Office of Population Census and Survey classifications) and birthing method (birth by caesarean versus non-caesarean birth).

Maternal education is coded into eight levels, one being the lowest possible educational achievement including individuals with poor or failing secondary school grades and level eight representing individuals with Doctoral degrees. Social class is broken down into five levels ranging from professionals (doctors, lawyers) to completely unskilled workers. Also included were maternal mental health history reported based on a series of yes or no questions in the period six months after birth (any versus no reported history of mental health problems), first trimester smoking (any versus none), and breastfeeding in the first two years of life (any versus none).

These covariates were selected based on their theoretical relationship to the outcome of interest. Being born to a mother below the age of 20 or to a family of low social economic status are commonly identified risk factors for children's mental health disorders (Peacock, Lewis, Northstone, & Wiles, 2011). Birthing method and breastfeeding behaviour have both been associated with the incidence of behavioural disorders and abnormal brain development in children (Rogers, Keating, Young, Wong, Licinio, Wesselingh, 2016) and may potentially confound the role of antibiotics in the development of behaviour disorders. Genetic studies have indicated many behavioural disorders may be caused in part by heritable genetic factors, highlighting the importance of controlling for maternal mental health concerns (Keller & Miller, 2006). The selection of possible confounders listed above is largely consistent with previous ALSPAC papers looking at behavioural outcomes based on the SDQ (Peacock, Lewis, Northstone, & Wiles, 2011).

Statistical analysis

The analysis began by describing the sample, in terms of the frequency of key exposures and outcomes and the proportion of missing data for each variable of interest. This was followed by an examination of the relationship between exposures and potential confounders using a chi-

square test of association. Given the high degree of missing data, a secondary descriptive analysis was conducted to explore any systematic differences between the analytic sample and those cases excluded from the study on all relevant variables using chi-square test of association. Findings were reported as counts and percentages, χ^2 and associated p-value.

A logistic regression model was built which included the three antibiotic exposure windows (0-5 months, 6-14 months, 15-23 months), the sex of the infant (male = 0, female = 1), and the dependent variable (SDQ total score at 81 months), classified into either normal/borderline (0) or abnormal (1) based on the age- and sex- specific abnormal range cut-points. This initial step was followed by the addition of a 'sex by exposure' interaction term for each period of exposure in a secondary step. When introduced in a stepwise fashion, these interaction terms were shown to significantly improve the model, as evidenced by the omnibus f-test statistic (p=.014) and the significance of the sex by antibiotic exposure at 0 to 6 months interaction term (p=.006) (Data not shown, see Appendix C). Accordingly, all subsequent models used in the final analyses were stratified by sex. Stratification is considered best practice in situations where the effect is negative, or extremely small in one of the two strata, as well as situations where the direction of the effect is opposite for the two strata (Behrens, Winkler, Gorski, Leitzmann, & Hedi, 2011).

Following stratification the final logistic regression model was built to explore the relationship between the antibiotic exposure windows and the outcome of interest, while controlling for potential confounders. The initial step of this model was a bivariate analysis including just the exposure and outcome, followed by a secondary step which included all covariates and potential confounders. This methodology is consistent with a previous study using the ALSPAC cohort examining associations between antibiotic exposure and obesity (Trasande,

Blustein, Liu, Corwin, Cox, & Blaser, 2013). Findings were reported as odds ratio (OR), 95% confidence interval (CI), p-value. SPSS 23 was used for all statistical analyses.

Missing data

After a comprehensive analysis of both the proportions and patterns of missing data, a complete cases analysis was decided upon as the analytical approach to reduce the risk of potential bias introduced by various other methods for dealing with missing data (e.g. mean substitution, last score forward, missing indictor method, and multiple imputation). It was clear from the missing data analysis that the data was not missing completely at random, as there were clear systematic differences between those with complete data and those with missing data. A comparison between the analytic sample and the excluded cases is shown in Table 2.

Ethics for research involving human subjects

This study did not require formal approval by the McMaster University Institutional Review Board because the study design was based on a secondary analysis of a de-identified dataset obtained under the ethics approval of the original study. Ethical approval for the original (parent) study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees.

Results

Description of the sample

Of the 3,468 children in the complete cases sample, 30.6% of the children received at least one exposure to antibiotics during the first 6 months of life, with the total number exposed increasing with age. By 24 months, 74.3% of the sample has been exposed to at least one course of antibiotics. In terms of other potential intestinal microbiome disruptors, 10.7% of the children were born by caesarean section and 20.5% were never breastfed. In terms of risk factors related

to mental health disorders, 50.8% of the sample were male, 9.7% were born to mothers with a self-reported mental health disorder, 1.1% were born to mothers under the age of 20, and 16.6% were born to mothers who smoked tobacco during at least the first trimester. By the age of 81 months, 10.2% of children were in the clinical range for behavioural problems. See Table 2 for the full descriptive analysis as well as a comparison to those excluded from the study. Additional descriptive analysis was conduced to describe the analytic sample by sex and exposure status. See appendix E and F for full data.

Generalizability analysis

Due to the large proportion of case excluded by list-wise deletion in the complete cases analysis, chi-square measure of association was used to compare those in the analytic sample and those excluded due to missing data to determine potential bias in the sample. Overall, children in the analytic sample were healthier and exposed to fewer risk factors for developing mental health problems than those excluded. Cases included in the analysis were significantly less likely to be exposed to smoking in the first trimester (16.6% compared to 28.1%, p <.001), born to a mother with a mental health disorder (9.7% compared to 12.9%, p <.001), or born to a mother under the age of 20 (1.1% compared to 5.8%, p <.001). Included cases were also more likely to be born to mothers with a higher education (highest educational achievement of 'degree', 15.6% compared to 11.8%, p =<.001), be of higher social class (manual labour-based family income, 6.1% compared to 8.7%, p =.001), and were more likely to have breastfed (79.5% compared to 73.4%, p<.001). There were no significant differences in terms of rates of caesarean section (p=.631) or the sex of the child (p=.267). Those in the analytic sample were slightly less likely to have been exposed to at least one antibiotic in the 0-5 months window (30.6% compared to 33%, p=.011); there were no statistically significant differences in the 6-14 months (p=.953) or 15-23 months

(p=.605) exposure windows. Those included in the analytic sample had significantly lower rates of behavioural disorders in the clinical range as measured by the SDQ (10.2% compared to 18.6%, p<.001). These differences suggest the analytic sample was substantially more socially advantaged, healthier, and less likely to experience the outcome of interest when compared to those excluded from the analysis. These differences may impact the generalizability of the findings of the study to the general population. See Table 2 for the full data along with chi-square measure of association results.

Factors associated with antibiotic exposure

No significant associations between antibiotic exposure in the first 24 months of life and the following potential confounders were identified, including maternal social class (p=.225), maternal education level (p=.373), tobacco use during the first trimester (p=.400), history of mental health problem (p=.123), age of mother under 20 at time of birth (p=.230), breastfeeding behaviour (p=.542), or caesarean section (p=.548). The only potential confounder significantly associated with antibiotic exposure was the sex of the child, where males were significantly more likely than females to be exposed to antibiotics (77.7% of males were exposed compared to 70.7% of females, p<.001).

Table 2: Sample Characteristics, by complete cases and excluded cases, with measure of association

Characteristic	Level	Complete cases analytic sample		Excluded by list-wise deletion		Chi-square test of association	
		Count	%	Count	%	X^2	P value
Sex	Male	1763	50.8	5454	51.9	1.233	.267
	Female	1705	49.2	5050	48.1		
One or more antibiotics 0-5m	Unexposed	2408	69.4	5335	67	6.389	.011
	Exposed	1060	30.6	2624	33		
One or more antibiotics 6-14m	Unexposed	1607	46.3	3523	46.4	.003	.953
	Exposed	1861	53.7	4070	53.6		
One or more antibiotics 15-23m	Unexposed	1837	53	3643	52.4	.268	.605
	Exposed	1631	47	3305	47.6		
Any antibiotics before 24m	Unexposed	893	25.7	2772	31.4	38.040	<.000
	Exposed	2575	74.3	6055	68.6		
Social Class – Maternal	I	243	7	347	5.3	57.069	<.000
	II	1109	32	2059	31.3		
	III (non-manual)	1556	44.9	2747	41.7		
	III (manual)	212	6.1	573	8.7		
	IV	275	7.9	717	10.9		
	V	73	2.1	145	2.2		
Moms highest education level obtained	CSE	360	11.2	2114	23.6	291.709	<.000
	Vocational	295	8.5	929	10.4		
	O level	1283	37	3010	33.7		
	A level	959	27.7	1833	20.5		
	Degree	541	15.6	1058	11.8		
Tobacco use in First Trimester	Unexposed	2893	83.4	6966	71.9	179.723	<.000
	Exposed	575	16.6	2719	28.10		
Maternal history of MH	Yes	337	9.7	1158	12.9	23.794	<.000
	No	3131	90.3	7828	87.1		
Age of mother at delivery <20	Yes	38	1.1	612	5.8	131.536	<.000
	No	3430	98.9	9892	94.2		
Any breastfeeding	Yes	2756	79.5	5625	73.4	47.921	<.000
	No	712	20.5	2043	26.6		
Born by caesarean	No	3096	89.3	7412	89.6	.230	.631
	Yes	372	10.7	863	10.4		
SDQ Total – Clinical	Yes	353	10.2	517	18.6	91.010	<.000
	No	3115	89.8	2265	81.4		

Abbreviations: m, months; MH, mental health; SDQ, Strength and Difficulties Questionnaire

Antibiotic exposures and behavioural outcomes

Univariate analysis

In the univariate logistic regression model an exposure to one or more antibiotics in the 15 to 23-month exposure window was significantly associated with an increased risk of developing behavioural disorder at 81 months in females (OR=1.527, 95% CI 1.048-2.225 p=.028), but not in males. While not statistically significant (p=>.05), males showed a decrease in risk following exposure in the 0 to 6-month and 7 to 13-month exposure windows. See Appendix D for the full univariate findings.

Multivariate analysis

In the multivariate model, which included all other covariates, the 6 to 14-month antibiotic exposure was shown to be significantly associated with reduced odds of developing abnormal behavioural problems in males (OR=.719, 95% CI=.530-.976, p=.034). In the multivariate model an exposure in the 15 to 23-month window remained significant for females (OR=1.549, 95% CI=1.055-.2.272, p=.025). In both the male and female models, first trimester smoking (p=<.001; p=.015), maternal mental health problems (p=.038; p=.006), were associated with an increased risk of behavioural disorders. Maternal age under 20 at birth significantly increased the odds of the outcome for males (p=.006), but showed no significant association in females (p=.215). In terms of social class, only Social Class (III) Non-manual was associated with a decreased risk of the outcome, and only in females (OR=.376, 95% CI=.156-.9.03, p=.029). Maternal education levels (p=>.05) and Breastfeeding (p=>.05) were unassociated with the outcome for either sex. Similar to antibiotic exposure at 15-23-months birth by Caesarean section was also significantly associated with the outcome in females, (OR=1.750, 95% CI=.1.062-.2.884, p=.028), but showed a non-significant decrease in risk in males (p=0.06). See table 3 for the full data.

Table 3: Multivariate Logistic Regression

Variables	Male								Female							
							95% C.I. for EXP(B)								95% C.I. for EXP(B)	
	B	S.E.	Wald	df	Sig.	Exp (B)	Lower	Upper	B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Antibiotic Exposure 0-5	-.287	.166	3.010	1	.083	.750	.542	1.038	.276	.206	1.786	1	.181	1.318	.879	1.975
Antibiotic Exposure6-14	-.330	.156	4.486	1	.034	.719	.530	.976	-.053	.199	.070	1	.791	.949	.642	1.402
Antibiotic Exposure 15-23	.052	.153	.117	1	.733	1.054	.780	1.423	.437	.196	4.998	1	.025	1.549	1.055	2.272
FirstTriTobacco	.680	.176	14.921	1	.000	1.973	1.398	2.785	.545	.224	5.929	1	.015	1.724	1.112	2.673
MaternalMH	.461	.222	4.303	1	.038	1.586	1.026	2.452	.690	.253	7.456	1	.006	1.993	1.215	3.270
Under20atDelivery	-.287	.166	3.010	1	.083	.750	.542	9.309	.845	.681	1.538	1	.215	2.327	.613	8.842
Breastfeeding	.135	.179	.572	1	.450	1.145	.806	1.625	.178	.228	.613	1	.434	1.195	.765	1.866
Caesarean	-.501	.273	3.369	1	.066	.606	.355	1.035	.560	.255	4.819	1	.028	1.750	1.062	2.884
Social Class			6.635	5	.249						6.709	5	.243			
Social Class (I)	-1.263	.646	3.820	1	.051	.283	.080	1.004	-.387	.619	.390	1	.532	.679	.202	2.285
Social Class (II)	-.288	.477	.364	1	.546	.750	.295	1.909	-.858	.475	3.271	1	.071	.424	.167	1.074
Social Class (III) Non-manual	-.193	.457	.179	1	.672	.824	.336	2.019	-.979	.448	4.785	1	.029	.376	.156	.903
Social Class (III)	-.297	.521	.326	1	.568	.743	.268	2.062	-.931	.558	2.784	1	.095	.394	.132	1.177
Social Class (IV)	-.537	.517	1.077	1	.299	.585	.212	1.611	-.610	.495	1.515	1	.218	.544	.206	1.435
Maternal Education			2.060	4	.725						5.462	4	.243			
Vocational	.206	.299	.473	1	.492	1.228	.683	2.208	-.479	.445	1.156	1	.282	.620	.259	1.483
O level	-.130	.245	.281	1	.596	.878	.543	1.420	.086	.285	.091	1	.763	1.090	.623	1.906
A Level	.023	.270	.007	1	.932	1.023	.602	1.738	-.455	.347	1.720	1	.190	.635	.322	1.252
Degree	.048	.329	.021	1	.885	1.049	.551	1.998	-.342	.433	.622	1	.430	.711	.304	1.660
Constant	-1563	.490	10.153	1	.001	.210		2.785	-2.133	.473	20.353	1	.000	.118		

Abbreviations: Tri, trimester; MH, mental health; Under20, Maternal age under 20

Discussion

This secondary analysis of the ALSPAC cohort found that exposure to antibiotics in specific windows within the first two years of life was associated with sex specific differences in the rates of behavioural disorders at 81 months. These effects persisted after controlling for potential confounders. Males exposed to antibiotics in the period between 6 and 14 months had significantly reduced odds of developing behavioural problems in the abnormal range at 81 months of age, while females exposed between 15 and 23 months of age had significantly increased odds of behaviour problems in the abnormal range. While many of the exposure-outcome associations did not reach statistically significance, an overall trend emerged that suggested a pattern of decreased risk of behavioural disorders in exposed males and an increase in risk in exposed females. These results are consistent with the DOHaD hypothesis which suggests that early life exposures to various stressors during critical developmental periods will have lasting impacts on an organism's health outcomes.

In terms of the other covariates, previously identified risk factors, such as maternal smoking during the first trimester and maternal history of mental health problems, were consistently associated with increased odds of behavioural problems in the abnormal range across both sexes. Interestingly, birth by caesarean section showed a similar pattern of sex-dependent effects of antibiotic exposure: reduced odds for males (OR=.606, 95%CI=.355-1.035, and increased odds for females (OR=1.75, 95% CI=1.062-2.884).

While sex dependent differences in the effects of microbiome disruption were expected based on the results observed in the GF rodent studies, it was hypothesized that a healthy and diverse intestinal microbiome would be adaptive and health promoting for the host organism, regardless of its sex. There is limited research to help explain why the behavioural effects of

microbiome disruption would be observed as adaptive in males and mal-adaptive in females, but there are few plausible explanations for these sex specific effects.

It is possible that these differences may be the result of underlying genotype – microbiome interactions which result in opposing effects in males and females. The observed differences may be the result of a complex interaction between sexual hormones present during sexual maturation during the post-natal period (Jasarevic, Morrison & Bale, 2016), or from well-established immunological differences between the sexes (Ngun, Ghahramani, Sanchez, Bocklandt & Vilain, 2011; Libert, Dejager & Pineiro, 2010). Controlled studies performed in mice have confirmed that androgens impact the composition of the microbiome in a top-down fashion (Yurkoveytskiy, et. al, 2013). Female immune systems also tend to respond to infection more aggressively than males, which can aid in survival, but may also contribute to higher rates of immunogenic disorders in females (Ngun, Ghahramani, Sanchez, Bocklandt & Vilain, 2011; Libert, Dejager & Pineiro, 2010). Current research into the neuro-immune axis has clearly identified linkages between inflammatory markers, which are strongly influenced by the microbiota, and behavioural disorders (Kraneveld, et al., 2014). If the immune reaction resulting from bacterial colonization varies by sex, so may the resulting behavioural effects.

It is also possible that the physiological changes in brain structure and function (increased levels of serotonin in the hippocampus, higher levels of tryptophan and serotonin present in their blood, altered production of BDNF, and altered myelinisation in the pre-frontal cortex) observed only in male GF mice (Hoban, et al., 2016; Clark, et al., 2013) manifest differently in human subjects and/or contribute differently to behavioural outcomes.

Like previous studies examining the relationship between microbiome disruption and various health outcomes, this study cannot determine causation due to a lack of randomization

and the potential for residual confounding. For this reason, it is important to view these results with a certain degree of skepticism. There is a potential that the effects associated with antibiotic exposure and caesarean section are in fact that result of another factor associated with both the exposure and outcome, such as an underlying medical condition necessitating the intervention. However, there is currently no theoretical justification for suggesting that the poor health status underling the needs for antibiotic exposure would reduce the risk of behavioural disorders in males and increase the risk in females, or that the conditions necessitating birth by caesarean section would have a positive effect on males and a significantly negative effect in females. It is more likely that there is in fact another sex dependent mechanism common to both - such as the gut-microbiome-brain axis.

Clinical Implications

There are important clinical implications for the findings of this study. If there is indeed a causal relationship between microbiome dysbiosis in infancy and increased risk for behavioural disorders, this may provide opportunities for interventions which may help to reduce these risks and provide targets for novel therapeutic approaches. For example, the potential impacts of early life antibiotic use may be reduced through the application of probiotic formulas immediately following antibiotic exposure, a practice becoming common in the treatment of various bacterial infections (Homan & Orel, 2015). The potential risks of caesarean section could be reduced through the application of vaginal swaps from the mother to the face and mouth of infancy born by caesarean section (Dominguez-Bello, et al., 2016).

The gut-microbiome-axis may also provide the opportunity for novel therapeutics for the treatment of behavioural disorders in children. Early research suggests that the applications of psychobiotics (Romijn & Rucklidge, 2015) and fecal transplantation may have the potential to

reduce the severity of mental health symptoms (Evrensel & Ceylan, 2016). Previous authors have caution that future research into these areas should be conducted with the highest standards of clinical research to ensure the reputation of the field damaged through the dissemination of spurious claims (Cryan & Dinan, 2012).

Limitations

The current study has several important limitations. Firstly, the exposure classification is based on parental self-report of the previous 6-9-month period, which is likely to contain a certain degree of inaccuracy and potential for recall bias (Coughlin, 1990). The exposure data is also quite limited in terms of dosage, administration method, and type of antibiotic, important variables that may influence the impact on the infant's intestinal microbiome. In addition, the observational study design creates the potential for biological, behavioural, social and confounding factors due to a lack of randomization and ability to isolate the effects of the specific variables of interest. While the current study utilized a rich set of covariates to attempt to control for potential confounding, it is possible that not all relevant characteristics were controlled for which may further attenuate the association between the exposure and outcome.

The proportion of missing data excluded in the complete cases analysis may limit the generalizability of the current study. Exploratory analysis identified relevant systematic differences between the analytic sample used in the current study and those excluded due to missing data. The analytic sample was exposed to significantly fewer risk factors for developing the outcome and had much lower baseline rates of mental health problems than those excluded from the study. These differences were also reported in a previous study which concluded that, while drop-out rates were comparable to similar previous studies, drop-out was significantly more common amongst families who had children with behavioural disorders (Wolke, et al.,

2009). The missing data analysis indicates that both those who experienced the exposure and those who experienced the outcome were at significantly higher risk of being excluded from the analytical sample. This resulted in a dataset with significantly less cases with the potential to demonstrate the association between exposure and outcome. It is possible that the bias introduced by this pattern of missing data may have decreased both the strength and significance of the observed exposure-outcome associations. Overall, it may also have more is more difficult to detect the relationship between the exposure and outcome in such an abnormally health sub-set of the cohort with higher than normal resilience to developing the outcome despite exposure. It is possible that observed effects might be more easily observed in an unbiased sample with more complete outcome data, suggesting these results may actually be underestimating the strength of the association.

Future research

Future research will likely benefit from adopting more reliable and valid measures of both the exposure and outcomes of interest to further explore potentially subtle effects on CNS functioning and varying responses based on the exposure type, duration and intensity. More detailed information concerning the antibiotic exposures to determine the exact antibiotic type, route of administration and dosage could be used to better isolate potential effects. It would also be helpful to have biological data, such a stool samples, to confirm the state of the microbiome both prior to and following the exposure to better understand the relationship between these changes and the accompanying behavioural changes. Future studies may also wish to expand the range of assessments beyond psychopathology and also collect data on additional facets psychological functioning, such as executive functioning, and language or motor development. Classifications of behavioural disorders could be more reliably measured using more rigorous

methods to classify the presence of psychopathology including full clinical interviews, multiple informants, and/or other direct observation.

Future research may also wish to create an index variable that combines the effects of multiple microbiome disruptors both within and across generations. For example creating a composite score which combines risk factors such as maternal use of antibiotics during pregnancy, birth by caesarean section, lack of breastfeeding, and exposures to antibiotics in infancy. It would also be interesting to explore the role of environmental stressors that may work synergistically with antibiotic induced dysbiosis to contribute to unfavourable mental health outcomes.

Conclusion

Like many important research questions explored by observational designs, this question is ill-suited for randomized controlled trials. It would be highly unethical to randomize children to receive unnecessary antibiotic exposures, caesarean sections, or lack of breastfeeding to determine the potential effects on psychological functioning. Currently studies are being conducted which will hopefully help to further clarify the mechanisms that may be driving these effects, as well as more carefully control for potential confounding through innovative research protocols.

Despite the numerous limitations these results suggest there may be a potentially important relationship between various types of microbiome disruption in infancy and changes in observable behavioural characteristics associated with psychopathology. It is clear from the animal models and these results that sex of the participant should be considered when studying the relationship between microbiome disruption and the resulting physiological changes and accompanying behavioural effects.

In conclusion, the current findings support the hypothesis that disruption of the microbiome in early life results in measurable, sex specific changes in behavioural characteristics associated with psychopathology. These early exploratory findings support further study into the mechanisms of potential behavioural effects of disruption of the human microbiome in early life through exposure to antibiotics or birth by caesarean section. If a causal relationship is determined between early life microbiome disruptors and the development of psychopathology, the relative effect of these disruptors in relation to other developmental processes needs further consideration, given the potential impact these findings may have on the utilization of these intervention in infancy and early life.

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Appendix A: Transformation of SDQ Total score

Explore: SDQ total difficulties score (prorated) - Raw

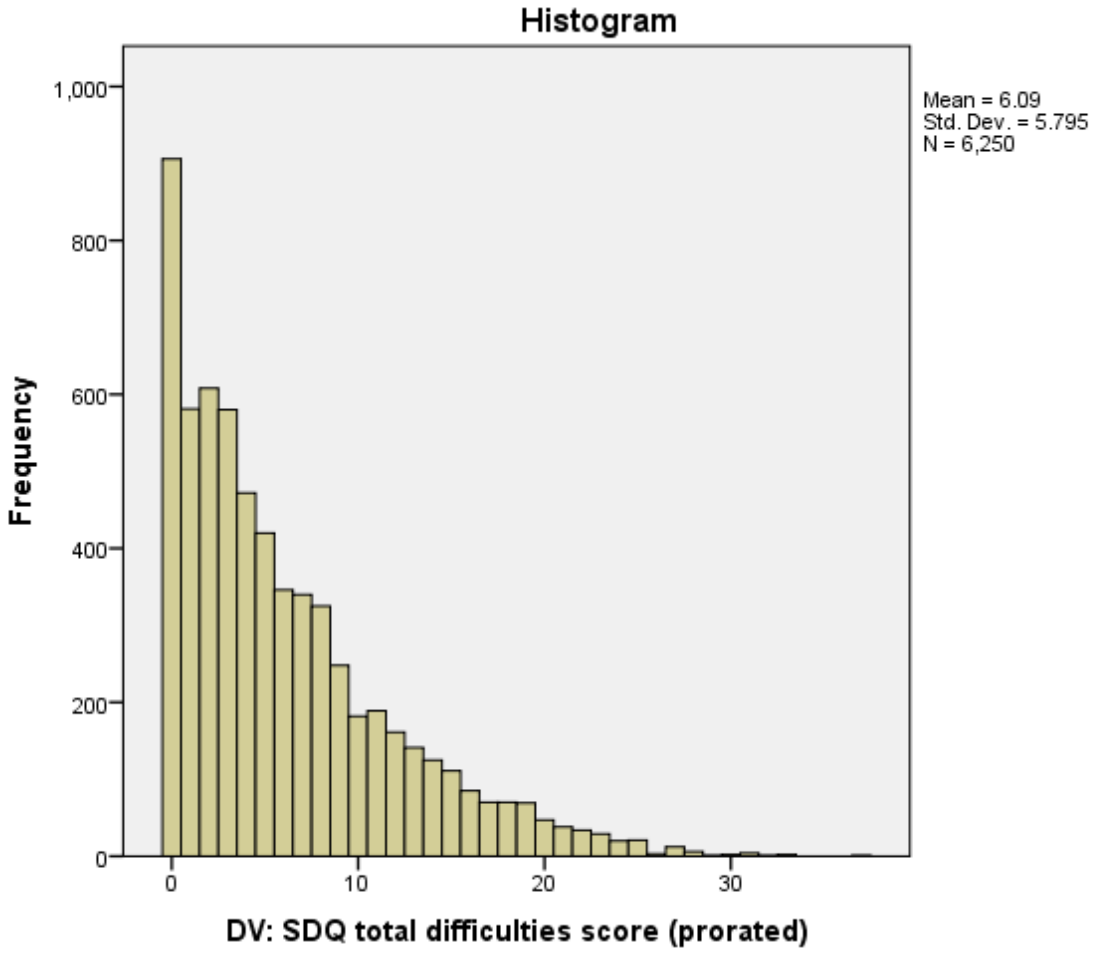
Descriptives

			Statistic	Std. Error
DV: SDQ total difficulties score (prorated)	Mean		6.09	.073
	95% Confidence Interval for Mean	Lower Bound	5.95	
		Upper Bound	6.24	
	5% Trimmed Mean		5.56	
	Median		4.00	
	Variance		33.579	
	Std. Deviation		5.795	
	Minimum		0	
	Maximum		37	
	Range		37	
	Interquartile Range		7	
	Skewness		1.255	.031
	Kurtosis		1.377	.062

Tests of Normality

	Kolmogorov-Smirnov[a]		
	Statistic	df	Sig.
DV: SDQ total difficulties score (prorated)	.146	6250	0.000

a. Lilliefors Significance Correction


Histogram
Mean = 6.09
Std. Dev. = 5.795
N = 6,250
Frequency
1,000
800
600
400
200
0
0
10
20
30
DV: SDQ total difficulties score (prorated)

Explore: SDQ total difficulties score (prorated) - SQRT Transformation

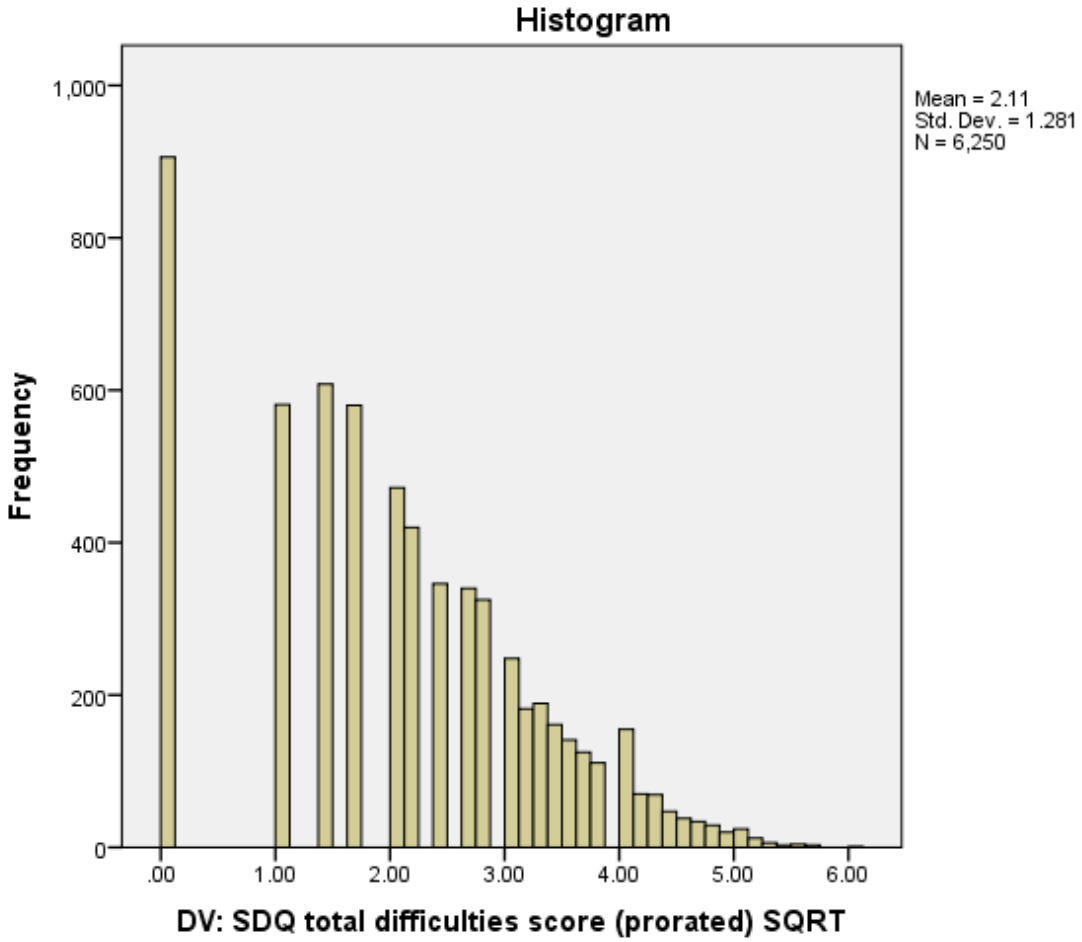
Descriptives

			Statistic	Std. Error
DV: SDQ total difficulties score (prorated) SQRT	Mean		2.1102	.01621
	95% Confidence Interval for Mean	Lower Bound	2.0784	
		Upper Bound	2.1419	
	5% Trimmed Mean		2.0862	
	Median		2.0000	
	Variance		1.642	
	Std. Deviation		1.28131	
	Minimum		0.00	
	Maximum		6.08	
	Range		6.08	
	Interquartile Range		1.59	
	Skewness		.029	.031
	Kurtosis		-.579	.062

Tests of Normality

	Kolmogorov-Smirnov[a]		
	Statistic	df	Sig.
DV: SDQ total difficulties score (prorated) SQRT	.095	6250	.000

a. Lilliefors Significance Correction


Histogram
Mean = 2.11
Std. Dev. = 1.281
N = 6,250
Frequency
1,000
800
600
400
200
0
.00
1.00
2.00
3.00
4.00
5.00
6.00
DV: SDQ total difficulties score (prorated) SQRT

Appendix B – Proportion of missing data on key variables for those sample alive at year one

Variable	Valid	Missing	% Missing
Sex	13972	0	0.0%
0-5 Antibiotic Exposure	11427	2545	18.2%
6-14 Antibiotic Exposure	11061	2911	20.8%
15-23 Antibiotic Exposure	10416	3556	25.5%
Any tobacco smoked during 1st 3mnths of Pregnancy	13153	819	5.9%
Any maternal history of mental health problems	12454	1518	10.9%
Age of mother at delivery <20	13972	0	0.0%
Social Class – Maternal	10056	3916	28.0%
Mums highest ed qualification	12412	1560	11.2%
DV: SDQ total difficulties score (prorated)	6250	7722	55.3%

Appendix C – Testing sex-exposure interaction

Block 2: Method = Enter

Omnibus Tests of Model Coefficients

		Chi-square	Df	Sig.
Step 1	Step	10.647	3	.014
	Block	10.647	3	.014
	Model	49.382	7	.000

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Sex	-.894	.162	30.303	1	.000	.409
	Antibiotics0to5	-.204	.133	2.354	1	.125	.815
	Antibiotics6to14	-.176	.129	1.861	1	.172	.839
	Antibiotics15to23	-.021	.126	.028	1	.868	.979
	Sex0to5	.591	.215	7.519	1	.006	1.806
	Sex6to14	-.039	.210	.035	1	.852	.962
	Sex15to23	.303	.205	2.181	1	.140	1.354
	Constant	-1.630	.102	256.150	1	.000	.196

a. Variable(s) entered on step 1: Sex0to5, Sex6to14, Sex15to23.

Appendix D - Univariate Model

Variables	Male								Female							
							95% C.I. for EXP(B)								95% C.I. for EXP(B)	
	B	S.E.	Wald	df	Sig.	Exp (B)	Lower	Upper	B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Antibiotic Exposure 0-5	-0.266	0.161	2.717	1.000	0.099	0.766	0.559	1.052	0.330	0.203	2.659	1.000	0.103	1.391	0.935	2.069
Antibiotic Exposure6-14	-0.287	0.152	3.566	1.000	0.059	0.750	0.557	1.011	-0.096	0.197	0.238	1.000	0.626	0.909	0.618	1.336
Antibiotic Exposure 15-23	0.057	0.150	0.145	1.000	0.703	1.059	0.790	1.419	0.423	0.192	4.849	1.000	0.028	1.527	1.048	2.225
Constant	-1.708	0.118	209.512	1.000	0.000	0.181			-2.766	0.158	307.577	1.000	0.000	0.063		

Appendix E: Analytic sample characteristics by sex			Sex		Total
			Male	Female	
One or more antibiotics 0-5m**	Unexposed	Count	1153	1255	2408
		%	65.4%	73.6%	69.4%
	Exposed	Count	610	450	1060
		%	34.6%	26.4%	30.6%
One or more antibiotics 6-14m**	Unexposed	Count	746	861	1607
		%	42.3%	50.5%	46.3%
	Exposed	Count	1017	844	1861
		%	57.7%	49.5%	53.7%
One or more antibiotics 15-23m*	Unexposed	Count	888	949	1837
		%	50.4%	55.7%	53.0%
	Exposed	Count	875	756	1631
		%	49.6%	44.3%	47.0%
Social Class – Maternal	I	Count	130	113	243
		%	7.4%	6.6%	7.0%
	II	Count	560	549	1109
		%	31.8%	32.2%	32.0%
	III (non-	Count	793	763	1556
		%	45.0%	44.8%	44.9%
	III (manual)	Count	110	102	212
		%	6.2%	6.0%	6.1%
	IV	Count	136	139	275
		%	7.7%	8.2%	7.9%
	V	Count	34	39	73
		%	1.9%	2.3%	2.1%
Moms highest education level obtained	CSE	Count	190	200	390
		%	10.8%	11.7%	11.2%
	Vocational	Count	161	134	295
		%	9.1%	7.9%	8.5%
	O level	Count	660	623	1283
		%	37.4%	36.5%	37.0%
	A level	Count	470	489	959
		%	26.7%	28.7%	27.7%
	Degree	Count	282	259	541
		%	16.0%	15.2%	15.6%
Tobacco use in First Trimester	Unexposed	Count	1473	1420	2893
		%	83.6%	83.3%	83.4%
	Exposed	Count	290	285	575
		%	16.4%	16.7%	16.6%
Maternal history of MH	Unexposed	Count	1597	1534	3131
		%	90.6%	90.0%	90.3%
	Exposed	Count	166	171	337
		%	9.4%	10.0%	9.7%
Age of mother at delivery <20	Unexposed	Count	1742	1688	3430
		%	98.8%	99.0%	98.9%
	Exposed	Count	21	17	38
		%	1.2%	1.0%	1.1%
Any breastfeeding	Unexposed	Count	1386	1370	2756
		%	78.6%	80.4%	79.5%
	Exposed	Count	377	335	712
		%	21.4%	19.6%	20.5%
Born by caesarean	Unexposed	Count	1580	1516	3096
		%	89.6%	88.9%	89.3%
	Exposed	Count	183	189	372
		%	10.4%	11.1%	10.7%
		Count	1538	1577	3115

SDQ Total – Clinical**	Normal	%	87.2%	92.5%	89.8%
	Clinical	Count	225	128	353
		%	12.8%	7.5%	10.2%

** P=<.000; * P=0.05

Appendix F: Analytic sample characteristics by exposure			Exposed to antibiotic		Total
			Unexposed	Exposed	
Social Class – Maternal	I	Count	51	192	243
		%	5.7%	7.5%	7.0%
	II	Count	270	839	1109
		%	30.2%	32.6%	32.0%
	III (non-	Count	426	1130	1556
		%	47.7%	43.9%	44.9%
	III (manual)	Count	51	161	212
		%	5.7%	6.3%	6.1%
	IV	Count	75	200	275
		%	8.4%	7.8%	7.9%
	V	Count	20	53	73
		%	2.2%	2.1%	2.1%
Moms highest education level obtained	CSE	Count	116	274	390
		%	13.0%	10.6%	11.2%
	Vocational	Count	77	218	295
		%	8.6%	8.5%	8.5%
	O level	Count	325	958	1283
		%	36.4%	37.2%	37.0%
	A level	Count	245	714	959
		%	27.4%	27.7%	27.7%
	Degree	Count	130	411	541
		%	14.6%	16.0%	15.6%
Tobacco use in First Trimester	Unexposed	Count	753	2140	2893
		%	84.3%	83.1%	83.4%
	Exposed	Count	140	435	575
		%	15.7%	16.9%	16.6%
Maternal history of MH	Unexposed	Count	818	2313	3131
		%	91.6%	89.8%	90.3%
	Exposed	Count	75	262	337
		%	8.4%	10.2%	9.7%
Age of mother at delivery <20	Unexposed	Count	880	2550	3430
		%	98.5%	99.0%	98.9%
	Exposed	Count	13	25	38
		%	1.5%	1.0%	1.1%
Any breastfeeding	Unexposed	Count	716	2040	2756
		%	80.2%	79.2%	79.5%
	Exposed	Count	177	535	712
		%	19.8%	20.8%	20.5%
Born by caesarean	Unexposed	Count	802	2294	3096
		%	89.8%	89.1%	89.3%
	Exposed	Count	91	281	372
		%	10.2%	10.9%	10.7%
SDQ Total – Clinical	Normal	Count	808	2307	3115
		%	90.5%	89.6%	89.8%
	Clinical	Count	85	268	353
		%	9.5%	10.4%	10.2%

** P=<.000; * P=0.05