ARE OUR FAVORITE DRUGS KILLING THE WRONG BUGS? THE IMPACT OF ANTIBIOTIC TREATMENT ON DIARRHEA AND GROWTH IN EARLY CHILDHOOD

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology.

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ABSTRACT

Elizabeth Tacket Rogawski: Are our favorite drugs killing the wrong bugs? The impact of antibiotic treatment on diarrhea and growth in early childhood
(Under the direction of Daniel J. Westreich)

Diarrhea is a recurring illness in childhood that is associated with malnutrition, stunted growth, and cognitive impairment. Children with diarrhea and other common childhood illnesses are frequently treated with antibiotics, often against recommendations. Antibiotic exposures early in life may increase susceptibility to infections and affect child growth through modifications of the gastrointestinal microbiota. We assessed the impact of antibiotic treatment on diarrheal risk and growth in a birth cohort from 2009 to 2013 of 497 children from semi-urban slums of Vellore, India.

We estimated the effect of antibiotic treatment for diarrhea on the timing of a subsequent episode using inverse probability of exposure-weighted Kaplan-Meier curves. We also estimated the effect of any early life antibiotic exposure on rates of diarrhea using negative binomial regression. Based on these results, we used the parametric g-formula to model the impact of hypothetical interventions to prevent unnecessary antibiotic exposures. To assess the impact on growth, we estimated the effects of antibiotic exposures in the first 6 months on height and weight z-scores using longitudinal general linear regression.

More than half of children were given at least one course of antibiotics in the first 6 months and more than half of these exposures were likely unnecessary. Antibiotic treatment of diarrhea was associated with reduced time to a subsequent episode, especially among younger

infants. In addition, the adjusted relative incidence rate of diarrhea from 6 months to 3 years of age was higher among children who received any antibiotics before 6 months compared to those who did not, especially among children who were no longer exclusively breastfed by 6 months. We estimated that preventing unnecessary antibiotic exposures before 6 months could substantially reduce the incidence of diarrhea in early childhood. There were no associations between early antibiotic use and growth in the first 6 months and from 6 months to 3 years.

Early life antibiotic exposure was associated with increased diarrheal risk, but had no association with growth. While antibiotics must be used for treatment when necessary, the potential for increased susceptibility to diarrhea should be considered when making treatment decisions.

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AAD	Antibiotic-associated diarrhea
AGE	Acute gastroenteritis
AIC	Akaike's information criterion
AOM	Acute otitis media
BMI	Body mass index
CDAD	*Clostridium difficile*-associated diarrhea
CHAD	Community health and development hospital
CHERG	Child Health Epidemiology Reference Group
CI	Confidence interval
CMC	Christian Medical College
DAG	Directed acyclic graph
DALYs	Disability adjusted life years
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FMT	Fecal microbiota transplantation
GALT	Gut-associated lymphoid tissue
GEE	General estimating equations
GEMS	Global Enteric Multicenter Study

GI	Gastrointestinal
HAZ	Height-for-age z-score
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICU	Intensive care unit
IMCI	Integrated Management of Childhood Illness
IQ	Intelligence quotient
IQR	Interquartile range
IRD	Incidence rate difference
IRR	Incidence rate ratio
KM	Kaplan-Meier
LCECU	Low cost effective care unit
LMICs	Low and middle-income countries
MAL-ED	Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health
MTD	Median time difference
MTR	Median time ratio
NFHS	National Family Health Survey
NNT	Number needed to treat
OR	Odds ratio
ORS	Oral rehydration salts
PCR	Polymerase chain reaction

QIC	Quasi-likelihood independence model criterion
RR	Risk ratio
rRNA	Ribosomal ribonucleic acid
RUTF	Ready-to-use therapeutic food
SD	Standard deviation
SES	Socioeconomic status
UHC	Urban health center
UK	United Kingdom
UNICEF	United Nations International Children's Emergency Fund
URI	Upper respiratory infections
US	United States
WAZ	Weight-for-age z-score
WHO	World Health Organization
WHZ	Weight-for-height z-score

CHAPTER I: SPECIFIC AIMS

Diarrhea is a universal and recurring disease during childhood with the highest burden in low and middle-income countries (LMICs). India has the most childhood deaths due to diarrhea compared to all other countries, with an estimated 200,000 deaths in 2010. These deaths accounted for 13% of all deaths in Indian children under 5 years of age [1]. The overall incidence of diarrhea in India is also high: 2.5 episodes per child year in the first 6 months of life and more than 3 episodes per child-year among ages 6-23 months [2]. Early childhood diarrhea is a risk factor for malnutrition, stunted growth, and cognitive impairment, and contributes to a cycle between malnutrition and increased susceptibility to infections [3–6].

Children with diarrhea in India are often treated with antibiotics despite the fact that diarrhea is almost always due to infections that do not respond to antibiotics. The World Health Organization correspondingly does not recommend routine use of antibiotics to treat diarrhea [7–10]. However, antibiotics have quickly become a common exposure early in life given their use for the treatment of diarrhea and other childhood illnesses like upper respiratory infections and otitis media. In the United States (US) and United Kingdom (UK), for example, approximately one third of children received antibiotics before 6 months of age [11,12].

Antibiotics affect the gastrointestinal microbiota, which is the complex population of microorganisms in the human gastrointestinal tract. Animal and small scale human studies suggest that antibiotics decrease the diversity of the microbiota, can cause long-term changes in microbiota composition, and result in increased susceptibility to the emergence of pathogens

[13–19]. Microbiota development is critical during infancy and early childhood, when diversity of organisms at this time is important for normal intestinal and enteric immune system development [20,21]. Further, the microbiota plays an important role in supporting nutrient absorption and other metabolic functions associated with growth [22,23]. Antibiotic exposures early in life, and especially during infancy, may cause the largest and most long-lasting perturbations to the microbiota, resulting in the greatest impact on related health outcomes during this period [24]. While longitudinal patterns of diarrhea through childhood have been well-characterized [25–27], the effects of antibiotic treatment on diarrheal risk and development among children in LMICs have not been studied.

We assessed the impact of antibiotic treatment among young children on diarrheal risk (Aim 1) and growth outcomes (Aim 2). We completed a secondary analysis of existing data collected in a cohort study of 497 children in semi-urban slums of Vellore, Tamil Nadu, India from 2009 to 2013 [28]. Field workers visited the homes of enrolled children twice-weekly from birth to 3 years of age and captured diarrhea incidence data based on a 3-day recall period. Height and weight were measured monthly. Children in the cohort had a high incidence of diarrhea (half had 4 or more episodes in the first 3 years of life), and a quarter of episodes were treated with antibiotics. This study provided highly detailed existing data on antibiotic exposures, diarrhea incidence and severity, and growth trajectories.

Specific Aim 1: Estimate the effect of antibiotic treatment on future diarrheal risk among children in the first 3 years of life.

Aim 1A: Estimate the effect of antibiotic treatment for diarrhea on the incidence of a subsequent diarrhea episode.

Hypothesis 1: Antibiotic treatment for diarrhea reduced the time to subsequent diarrhea.

Aim 1B: Estimate the effect of any early life antibiotic exposure on rates of diarrhea from 6 months to 3 years of age.

Hypothesis 2: Children exposed to antibiotics early in life had increased rates of diarrhea from 6 months to 3 years of age compared to children who are not exposed to antibiotics before 6 months of age.

Aim 1C: Evaluate the impact of realistic interventions which prevent unnecessary antibiotic exposures early in life on rates of diarrhea from 6 months to 3 years of age.

Hypothesis 3: Interventions that remove unnecessary antibiotic exposures would have a substantial impact on reducing diarrhea incidence and would make an important contribution to public health.

Specific Aim 2: Estimate the effect of early life antibiotic exposures on short-term (0-6 months) and long-term growth (6 months to 3 years).

Hypothesis 3: Children exposed to antibiotics early in life had different growth rates compared to children not exposed to antibiotics, both in the short and long-term.

Understanding the impact of widespread antibiotic treatment among children is important for making treatment decisions and may support efforts to encourage rational antibiotic use. This

study also contributes epidemiologic evidence to the rapidly accumulating laboratory data which suggest that antibiotic-mediated changes to the microbiota may affect susceptibility to disease.

CHAPTER II: BACKGROUND AND SIGNIFICANCE

Burden and epidemiology of early childhood diarrhea

Burden

A large proportion of global child morbidity and mortality from infectious diseases is due to acute diarrheal diseases. Based on inconsistent data quality and quantity from around the world [29], estimates of child mortality due to diarrhea in the first 5 years of life from 2010 range from 6.8 to 14.3 million. The majority of these deaths are due to infectious causes, with diarrhea causing 600,000 to 1.4 million of those deaths [1,30–34]. In 2013, an estimated 6.3 million children died in their first 5 years, with diarrhea causing 578,000 of those deaths [35]. The burden of mortality is highest in Africa and Asia, and India has had the most childhood deaths due to diarrhea compared to all other countries. Diarrhea caused an estimated 334,000 deaths in Indian children in 2005, corresponding to 1 in 82 Indian children dying from diarrhea in the first 5 years of life [36,37], and more than 200,000 deaths in 2010 [1]. These deaths comprised 13-14% of all deaths in Indian children under 5 years of age, approximately 10% of deaths in infants (0-11 months) and 24% of deaths in children 1-4 years of age [1,36,38]. Other researchers have estimated that up to one third of Indian childhood deaths are due to diarrhea based on data from 2008 [39]. Because more than 70% of deaths from diarrhea occur in the first 2 years of life, interventions targeted in early childhood may have the largest impact on mortality [30].

As oral rehydration therapy for diarrhea has become more widespread, deaths due to diarrhea have declined dramatically over the last 20 years. The absolute number has declined by more than half from 1990 to 2010 despite increases in population size [31,40]. In the first decade of the 21st century, deaths due to diarrhea dropped by almost 400,000 [1]. However, while gains have been made in mortality, the incidence of acute diarrheal illnesses and associated morbidity remains high. Less than 2% of diarrhea episodes progress to severe disease, and the case fatality rate for severe diarrhea is only approximately 2% [30,41]. Given the substantial number of deaths due to diarrhea, the denominator of these proportions, corresponding to the total number of diarrhea episodes, is very large.

The global incidence of diarrhea in the first 5 years of life in 2010 was estimated to be 2.7 episodes per child-year, which corresponds to 1.7 billion episodes in 2010 [30]. Incidence is highest at age 6-11 months (4.5 episodes per child-year) and then decreases and levels off after two years of age (2.3 episodes per child-year) [2,40]. In India, diarrhea incidence in 2010 was estimated to be 2.50 episodes per child year in the first 6 months of life, and 3.82, 3.09, and 1.98 episodes per child year in ages 6-11 months, 12-23 months, and 24-59 months respectively [2]. The third National Family Health Survey (NFHS-3), a nationally representative household survey of over 100,000 households completed in 2005-2006 sponsored by the Ministry of Health and Family Welfare, Government of India, found that 9.0% of children under the age of 5 and 18.1% of children 6-11 months reported diarrhea in the 2 weeks prior to survey [7,8]. The coverage evaluation [42] and 10-district [43] surveys by the United Nations International Children's Emergency Fund (UNICEF) conducted in India in 2009 similarly estimated that 14.3% of Indian children aged 0-23 months and 19.8% of Indian children aged 2-59 months from the 10 districts had diarrhea in the 2 weeks prior to the two surveys respectively. The estimate

was 15.3% for the state of Tamil Nadu in the coverage evaluation survey [42] and 28.4% for the sampled Krishnagiri district of Tamil Nadu in the 10-district survey [43].

The mean duration of diarrhea episodes assessed in the community in LMICs has been estimated to be 4.3 days. In India, median duration was estimated to be 3 days in the UNICEF 10-district survey [43]. The majority of diarrhea cases (65%) are mild, with only 5% becoming persistent (lasting 14 days or more). Severe cases have longer duration (8.4 days) and high prevalence of dehydration [41]. Estimates of the proportion of diarrhea episodes with blood in the stool range from 1 to 12% [7,8,43].

The estimated cost to households per diarrhea episode was estimated to be US$ 6.47 in India, the majority of which covered direct medical costs. Given the high incidence rates of diarrhea, these costs aggregate to billions of dollars globally [44].

Etiology

A wide range of microbes cause childhood diarrhea, including bacteria, parasites, and viruses. The relative frequencies of pathogens associated with diarrhea vary by geography, season, child's age, breastfeeding practices, hygiene practices, immunocompetence, and secular time trends. The methods for pathogen testing, for example by culture or polymerase chain reaction (PCR), and the number of pathogens tested for in each study also influence prevalence estimates. Determination of etiology is complicated by co-infections, carriage of multiple potential pathogens, and the inability to identify pathogens in a third of cases [34,45]. Rotavirus, *Cryptosporidium, Shigella*, and enterotoxigenic *Escherichia coli* (ETEC) are most responsible for diarrheal diseases globally, and specifically for moderate-to-severe diarrhea in children [31,46]. Other *E. coli* virotypes, including enteroaggregative (EAEC), enteropathogenic (EPEC),

enteroinvasive (EIEC), and enterohaemorrhagic *E. coli* (EHEC), as well as *Campylobacter jejuni*, *Vibrio cholera* O1 and O139, and *Salmonella* are also regularly identified as bacterial causes of diarrhea in different regions of the world. *Giardia duodenalis*, *Entamoeba histolytica*, and *Cryptosporidium* are the common protozoal causes [10].

The Global Enteric Multicenter Study (GEMS) in 2007-2011 identified that approximately one quarter of moderate-to-severe diarrhea episodes in the first two years of life were attributable to rotavirus in Kolkata, India. *Cryptosporidium* was attributed with 7-12% of episodes. In older children aged 24-59 months, moderate-to-severe diarrhea was more often attributed to *Shigella* and rotavirus [46]. A multicentric hospital-based study in 1991 detected *Shigella* in 20% of acute diarrheal cases among children aged 0-35 months in Vellore, India, rotavirus in 18%, enterotoxigenic *E. coli* in 14%, and *Campylobacter jejuni* in 15%. However, these organisms were also isolated in relatively high frequencies from control children without diarrhea [47]. A more recent study in Kolkata isolated rotavirus (48%), *E. coli* (19%—including enteroaggregative *E. coli* in 12%), *Vibrio* (19%—including *V. cholera* O1 in 16.4%), *Giardia* (14%), adenovirus (12%), and *Cryptosporidium* (11%) from hospitalized children with diarrhea under 5 years of age [48]. Overall among hospitalized cases of gastroenteritis in India, rotavirus has been identified as the cause of 6-45% (median approximately 20%) of episodes, with other viruses such as caliciviruses (includes norovirus and sapovirus), adenovirus, and astrovirus contributing to a lesser extent [34,49–52]. The proportion of symptomatic cases attributable to rotavirus in the community is lower, approximately 15% (range 4-30%) [50].

Enteric pathogens most responsible for diarrhea mortality are rotavirus, *Vibrio cholerae*, *Shigella*, *Salmonella*, and *E. coli* [45]. A recent review of global diarrhea mortality completed by the Child Health Epidemiology Reference Group (CHERG) estimated that 55% of diarrhea

deaths were due to either rotavirus, EPEC, caliciviruses, or ETEC based on etiologies of hospitalized cases [34]. Bacterial and parasitic causes of diarrhea confer higher risk of diarrhea persistence and mortality when compared to viral infections [53–55]. However, given the high prevalence of rotavirus-associated diarrhea, estimates from 2008 suggest that 37% of diarrhea deaths in children were due to rotavirus [56]. The Million Death Study estimated 113,000 deaths in Indian children under the age of 5 were due to rotavirus in 2005, 4.14 deaths per 1000 live births. The rotavirus mortality rate estimated for Tamil Nadu was lower, 2.1 deaths per 1000 live births, with an estimated 2,400 deaths in 2005 [37]. Several diarrheal diseases, such as cholera, shigellosis, and typhoid, are also of special importance given they have been closely associated with extreme poverty due to their association with contaminated water and lack of sanitation and hygiene among the poorest populations [45].

Risk factors

Transmission of pathogens associated with acute diarrhea is seasonal, with peak incidence of most diarrheas in the wet season [26,57,58]. Heavy rainfall events in Ecuador have been linked to increases in diarrhea incidence following dry periods and decreases in diarrhea following wet periods, suggesting climate vulnerability may be common in areas with insufficient water treatment infrastructure [59]. Increases in ambient temperature are also associated with increases in diarrhea [58,60]. Conversely, rotavirus diarrhea, which is not associated with transmission through water, often peaks in the cold, dry season [61,62].

Diarrhea incidence rates also vary with age through the first 5 years of life. Children under 6 months of age are partially protected by breastfeeding through nutrients and maternal antibodies in breast milk. Peak incidence of diarrhea is seen in the months following weaning,

during the remainder of the first and the second year of life. Risk of persistent diarrhea is also highest at this young age [63]. Diarrhea incidence then decreases and stabilizes in the next 3 years of age [2,25,27,57]. Diarrheal risk is also dependent on previous episodes, such that risk is highest among children with recent, prolonged (duration 7-13 days), or persistent (duration ≥ 14 days) diarrhea [53,54,64–69]. Risk decreases as time elapses since the last diarrhea episode [25–27]. A longitudinal study of persistent diarrhea in a birth cohort from Brazil described increased burdens of acute diarrhea 3 months before and 18 months after episodes of persistent diarrhea [27]. Concurrent or recent non-diarrheal illnesses, such as pneumonia, intestinal parasitic infection, and positive blood culture, increase risk of death among children with diarrhea [25,55,65,70].

Malnourishment, as assessed by anthropomorphic measurements, is a risk factor for diarrhea incidence as well as poor outcomes such as persistence and death [53,55,65,70–73]. Micronutrient deficiencies, especially for zinc and vitamin A, increase risk of diarrhea and persistence of episodes [63,74,75]. Vitamin A deficiency compared to other nutritional deficiencies is most consistently associated with increased frequency, severity, and/or fatality of almost all infectious diseases. Most other vitamin deficiencies are also synergistic with infectious disease under some conditions, especially among malnourished individuals [74].

Breastfeeding, and specifically exclusive breastfeeding, is a well-known protective practice against diarrhea. Breast milk is a hygienic and rich source of nutrition and includes immune system components such as antibodies, lymphocytes, macrophages, lysozymes, and lactoferrin, which protect infants from gastrointestinal infections [76]. Children who are not exclusively breastfed or are weaned early have a one to two-fold increase in risk of acute diarrhea, persistent diarrhea, and diarrhea-associated death [54,57,70,72,73,77–83].

Male children in India have shown slightly higher prevalences of diarrhea than female children in national surveys [8,42,43]. Other host genetic factors also contribute to susceptibility to infection. Certain alleles of the genes encoding the histo-blood group antigens, which function as receptors for norovirus infection, have been found to prevent infection in some individuals [84]. Similarly, variants of the histo-blood group antigens, Lewis genes, and secretor genes mediate susceptibility to rotavirus infection [85,86]. Genes associated with the immune response, such as polymorphisms at the human leukocyte antigen (HLA) locus may modify an individual's ability to present and recognize microbial antigen. Variations in inflammatory response and presence of host receptors for pathogens may also affect the outcome of pathogen exposure [45]. For example, anergy and delayed hypersensitivity responses to standard skin-test antigens have been shown to increase diarrheal risk and duration in several studies [87]. Finally, an allele of the ApoE cholesterol transport protein has been shown to reduce the impact of diarrhea and malnutrition on cognitive impairment [45].

Factors associated with fecal-oral transmission of disease, such as hygiene practices and water quality, have been repeatedly associated with diarrheal risk. Hygiene practices around defecation, such as lack of latrine or toilet usage, improper disposal of feces, and lack of hand washing after defecation, have been associated with diarrhea incidence and duration in children in studies from Africa, South America, South Asia, and Southeast Asia [25,57,72,75,77,88–93]. Inconsistent maintenance of latrines and lack of education about their proper use may contribute to diarrheal risk even when improved sanitation facilities are present [94]. Unsafe or inadequate water sources also increase risk for diarrhea, specifically open storage of water, use of open water compared to pipe borne water, and consumption of water without boiling [72,73,77,88,91,95–99]. Similarly, behaviors related to food preparation and disposal, including

irregular food preparation, lack of hand washing before preparation, open storage of food, consumption of raw food, use of dirty milk bottles without cleaning with soap and hot water, and improper garbage disposal, have been associated with diarrhea in children [77,82,89–91,100,101]. Higher levels of contact with others who could transmit pathogens through day care attendance, crowding in the home, and exposure to domestic animals has also been associated with increased diarrheal risk [69,71,72,82,89,90,101,102]. Finally, recent or concurrent diarrhea episodes in other members of the household predict diarrheal illness in children [89,103].

While diarrhea affects all classes in society, highest morbidity and mortality occurs among the poor, and social factors have a large impact on diarrhea burden. Low socio-economic status and level of education of mothers is associated with diarrhea incidence, severity, and mortality [25,54,73,80,88,90,91,98,101,104]. Conversely, mothers' knowledge about the infectious spread of diarrhea and preventive measures are protective [88,91].

Longitudinal studies of childhood diarrhea have incorporated hierarchical and random effects modeling techniques to simultaneously assess proximal and distal causes of diarrhea incidence and duration. A study among children aged 0-36 months in northeastern Brazil was analyzed using an effect decomposition strategy to explain hierarchical relationships among risk factors for diarrhea, including socioeconomic status, sanitary and living conditions, child and care related-data, hygiene behavior, intestinal parasitic infections, and disease history indicators. The authors report direct effects of poor sanitation conditions and child and care-related variables such as prenatal examination during antenatal care visits, height-for-age z-score, and intestinal parasitic infections on increased risk for diarrhea. The observed effect of low socioeconomic status on diarrhea incidence was mainly mediated by lack of sanitation, inadequate neighborhood infrastructure, and poor housing conditions. Poor sanitation conditions

had the largest effect among children aged 13-36 months [25]. Socioeconomic status was also highly associated with duration of diarrhea, an effect also likely mediated by environmental conditions and hygiene behaviors [75].

Interventions to improve water, sanitation, and hygiene have demonstrated improvements in both diarrheal rates and longer-term morbidity, including growth and cognitive development [105]. For example, a meta-analysis of hand washing interventions found an overall 32% reduction in diarrhea burden associated with the interventions in LMICs [106]. Similarly, a study of the effect of a city-wide sanitation program on diarrhea prevalence found that improved connection to a sewerage system reduced diarrhea prevalence among children under 3 years of age in Brazil [107]. Improved sanitation reduced the association between poverty and diarrhea in this area, further supporting the evidence that diarrhea is associated with low socioeconomic status through poor sanitation and environmental conditions among the poor [108]. However, interventions to improve water, sanitation, and hygiene must be appropriately implemented to meet local needs. Latrine promotion and construction alone, for example, without education and consistent use may not be effective, as was the case in a rural sanitation program in Odisha, India [94]. Overall, these studies suggest age, nutritional status, and recent diarrhea burden are important host risk factors for diarrhea, while sanitation and hygiene have the largest impact on diarrheal risk among the environmental factors.

Diarrhea and growth

Malnutrition is both a risk factor and outcome of diarrhea, resulting in a bi-directional relationship between increased susceptibility to infection and poor growth outcomes. This often termed, “vicious cycle,” was first characterized in the 1960s and was heavily studied in the

1970s and 1980s [109,110]. Studies of the cyclical association between diarrhea and growth have been discussed in great detail and are complicated by issues of temporality, confounding by host and environmental factors, and modification of effects by age, growth parameter analyzed, and duration of follow-up [3,5,109,111–113]. The cycle between enteric infection and malnutrition in children is associated with intestinal damage, malabsorption, and impaired immune response. Outcomes of the cycle include growth failure and decreased fitness and cognitive function. Several opportunities for intervention to interrupt components this cycle are available, including drugs and vaccines against enteric pathogens, nutrient supplementation, modification of the microbiota, and interventions towards clean water and sanitation [114].

Undernourishment, as measured by height and weight measurements below the international growth reference standards, has been associated with an increase in diarrhea incidence and duration in the two months to one year following anthropomorphic measurement. Multiple studies have demonstrated an increase in duration of diarrheal disease in undernourished children [6,54,68,111,115–118]. For example, weight-for-age z-scores below -3 were associated with an approximate doubling of average duration of diarrhea in the following two months among children under 5 years of age in Brazil [115]. Effects of malnutrition on diarrhea incidence are less pronounced, with effect sizes (e.g. risk ratios) for the association between undernourishment and diarrhea incidence between 1 and 2 [68,115,119–122]. Several studies have reported no association between weight and/or height and diarrhea incidence, often when controlling for confounding by socio-economic status indicators [116,117,119]. For example, an intervention that gave a daily lipid-based nutrient supplement to Haitian infants aged 6–11 months in an urban slum did not reduce diarrhea prevalence despite improvements in linear growth [123]. As etiologic information on diarrhea episodes has become more common, studies

have explored the effect of malnutrition on etiology-specific diarrhea episodes, and results remain mixed. Weight and height-for-age z-scores were not associated with *Giardia* infection in the first 3 years of life in Peru [124]. In Bangladesh, weight-for-age z-scores below -2 were associated with incidence of diarrhea due to ETEC, *Cryptosporidium* sp., and *Entamoeba histolytica*, but not other bacterial or viral diarrheas in children 2-5 years of age [125].

Growth failure as an outcome of diarrhea has been thoroughly characterized in the last 5 decades. Early documentation of growth charts showed that while children in LMICs may follow average growth trajectories in the first 6 months of life while breastfeeding, growth stalls and children fall below the average growth curve as repeated episodes of diarrhea and other infections accumulate [109,111,112,126,127]. Subsequent studies have corroborated the evidence towards an effect of diarrhea on malnutrition in South America, South Asia, and Africa [27,54,57,117,128–150]. The associations are nuanced by definition of exposure (prevalence versus incidence of diarrhea) and duration of effects (long versus short term). Measures of longitudinal prevalence (proportion of time spent with diarrhea) have demonstrated the largest effects on growth parameters and are often used as the most relevant predictors of growth outcomes [144]. Evidence that prolonged and persistent episodes of diarrhea have larger impacts on weight and height compared to acute episodes (<7 days) support the conclusion that total time with diarrhea is an important predictor of growth [54].

Many early studies focused on the short-term impact of diarrhea on growth, such as effects on anthropomorphic measurements 1 to 3 months following diarrhea ascertainment. Short-term effects of diarrhea on weight [27,54,57,117,128,130,131,133,134,136,139,142,144,148,150,151], and to a lesser extent on height shortfalls [27,54,57,128–130,133–135,139,142,148,150], have been consistently

demonstrated. These results have been challenged by the hypothesis of "catch-up growth," such that rebounds in growth during and after convalescence negate a majority of the short-term effects of diarrhea. Several studies confirm that effects on growth may be transient [124,133–135,137,144,145,152], while others have found that effects sustain for up to several years [117,128,137,138,140,143,147,149]. Opportunity for catch-up growth is modified by age, nutritional status, the pathogen causing diarrhea, and burden of recurrent diarrhea and other common illnesses [113,153,154]. Younger children often face the most serious growth shortfalls, and malnourished children of low socioeconomic status (who face higher burden of illness overall) may be less able to recover from growth deficits [3,118,134,135].

A recent meta-analysis of 7 cohort studies in Peru, Brazil, Guinea-Bissau, and Bangladesh demonstrated short-term (1 month) associations of diarrhea prevalence with weight at all ages under 24 months. While weight shortfalls were transient, effects on height were more apparent in the long-term, as children with average or greater diarrhea burdens were 0.38 cm shorter than children without diarrhea at 24 months of age [4]. Similarly, a multi-country analysis identified linear associations between both cumulative diarrheal incidence and longitudinal diarrhea prevalence with the log odds of stunting (height-for-age z-score $\leq$ -2) at 24 months of age. Specifically, the pooled odds of stunting increased by 16% (95% confidence interval (CI): 7, 25) for every 5% increase in longitudinal diarrhea prevalence [155].

Biological mechanisms for the effect of diarrhea on growth involve reductions in nutrient availability due to direct loss and intestinal malabsorption, increased metabolic needs, tissue degradation, and decreased nutrient intake due to disease-induced anorexia or withholding of food [111,156–159]. Healthy absorptive function of the intestinal tract is most important in the first few years of life when nutrients are needed for normal growth and development of the brain

[45]. Sustained exposure to pathogens can cause environmental enteropathy (or tropical/environmental enteric dysfunction), a frequently subclinical condition termed the "impoverished gut," which is associated with living in poor unhygienic environments and results in impaired function and structure of the small intestine. The intestinal villi of people with environmental enteropathy have reduced surface area such that they are decreased in height or even flat, resembling a flatter leaf-like structure rather than the normal fingerlike structure. These changes reduce the ability to absorb nutrients, such as sugars, nitrogen, fats, and micronutrients [3,111,113,160–164]. Studies of infants in tropical countries have shown that these changes in villus architecture occur during the first few months of life, which suggests malabsorption and associated growth failure among children in these areas begins at an early age [163]. In a study of largely malnourished Indian children with chronic diarrhea, almost three-quarters showed abnormal histology of the jejunum and approximately two-thirds showed atrophy of villi [165].

In addition, chronic intestinal inflammation associated with environmental enteropathy leads to elevated immune response and increased permeability of the intestinal tract, which allows pathogens to more easily cross the intestinal barrier [3,111,113,160–163]. Continuously high levels of cytokines and increased blood leptin concentrations may also contribute to the suppression of appetite associated with disease [113]. Indicators of intestinal permeability, such as the lactulose:mannitol urinary excretion ratio, and indicators of chronic immunostimulation, such as fecal lactoferrin, have been associated with growth faltering in animal models and human studies [3,45,111,113,163,166–168]. For example, the highest values of an enteric enteropathy score, based on fecal levels of alpha-1-antitrypsin, neopterin, and myeloperoxidase, were associated with linear growth deficits of about 1 cm over 6 months in the first year of life among children across several LMIC sites [169]. Nutrition interventions aimed at strengthening the

immune system, improving mucosal barrier function, and compensating for malabsorption have been able to offset the negative effects of diarrhea on growth, indicating the importance of gastrointestinal health in mediating the relationship between diarrhea and malnutrition [3,5,111,113,170].

The effects of diarrhea on growth have been extended to effects on cognitive function, school performance, fitness, and chronic disease later in life. Longitudinal diarrhea prevalence has been negatively associated with cognitive outcomes such as intelligence quotient (IQ), age at starting school, appropriateness of age for the current school grade, and other cognitive tests [140,171–175]. The association between diarrhea and cognitive function is likely not a direct effect, but mediated by poor growth outcomes associated with diarrhea [172]. Diarrhea in the first two years of life has also been correlated with reduced fitness at 6–9 years of age as assessed by the Harvard Step Test [175]. In the last few years, links have also been made between early childhood diarrhea and metabolic syndrome later in life. Increased diarrhea burdens and associated growth faltering are followed by an increase in risk factors for cardiovascular disease, such as dyslipidemia, hypertension, and glucose intolerance, several decades later [3,176,177].

The sum of evidence points towards substantial long-term effects of childhood diarrhea on gastrointestinal function, malnutrition, growth, cognition, and risk for chronic disease. These findings have prompted several researchers to recommend updating the calculations for diarrhea disability adjusted life years (DALYs) to include long-term morbidity associated with diarrhea [178,179]. Interventions that improve nutrition and reduce diarrheal disease burden may impact multiple elements of the complex relationships among these outcomes.

Available and recommended treatments

Improved treatment of acute diarrhea in children with oral rehydration therapy has been largely responsible for the drops in diarrhea-related mortality in children under 5 in the last few decades [45]. The World Health Organization (WHO) recommends that treatment of diarrhea involve three main tenants: fluid replacement to prevent or treat dehydration, zinc supplementation to reduce severity and duration of the episode, and continued feeding to prevent malnutrition [10]. Caregivers should give more fluids than usual, ideally appropriate homemade fluids containing salt (referred to as recommended home fluids), to children with diarrhea but without signs of dehydration [10,180]. Oral rehydration therapy with oral rehydration salts (ORS) solution given orally and intravenously is preferred for addressing moderate and severe dehydration respectively. The currently recommended ORS solution is a low osmolarity mixture of glucose and several salts dissolved in water [10]. ORS alone can effectively treat 90% of diarrheas with some dehydration. ORS containing cooked rice powder instead of glucose may provide additional benefit by reducing the rate of stool output [181]. Children should be fed a normal diet appropriate for their age throughout the episode, including breastfeeding for young children [10].

Zinc supplementation (10-20 mg/day) for 10 to 14 days is recommended for all children with diarrhea. Supplementation replaces zinc lost during diarrhea and reduces risk of a subsequent diarrhea episode in the following 2 to 3 months [10,182]. A Cochrane review of trials for zinc supplementation during diarrhea concluded that zinc reduces duration of acute and persistent diarrhea among children greater than 6 months of age, especially among children with signs of moderate malnutrition. However, this effect may be reversed among young infants [183]. Zinc also reduces the proportion of children with diarrhea persisting more than 3 and 7

days, and may have a protective effect against hospitalizations and death [183–185]. Unfortunately, the uptake of this recommendation has been suboptimal in many settings.

Antidiarrheal drugs, such as adsorbents, antimotility drugs, and bismuth subsalicylate, have no practical benefit and can be dangerous in children. Therefore, they should never be given for treatment of acute diarrhea. Anti-protozoal drugs are also rarely indicated [10]. Recently, the antisecretory drugs racecadotril and diosmectite have showed some evidence of reducing stool output and duration, though contradictory results have also been reported and these drugs are not recommended in India [186–188].

A large variety of probiotic formulations are available as supplemental treatment for diarrhea. However, a minority of strains have been found to conclusively provide benefit, specifically *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii*, which both reduce duration of diarrhea. Optimal timing and dosing of probiotic treatment is unknown. In addition, randomized control trials of probiotics have almost all been completed in populations from high-income countries, and effectiveness in LMICs has not been demonstrated [186,187]. Treatment guidelines in India do not recommend probiotics given the lack of evidence in Indian populations [188].

Antibiotics

Antibiotics are not recommended for routine treatment of acute or persistent diarrhea, except for acute bloody diarrhea (dysentery) and suspected cholera with severe dehydration. Because acute bloody diarrhea is likely to be caused by *Shigella*, children should receive ciprofloxacin for 3 days or another antibiotic effective against local *Shigella* such as ceftriaxone or pivmecillinam for 5 days. Many common antibiotics are ineffective for treatment of

shigellosis and should not be considered, including metronidazole, streptomycin, amoxicillin, tetracyclines, chloramphenicol, sulfonamides, hydroxyquinolines, nitrofurans (nitrofurantoin, furazolidone), aminoglycosides (neomycin, gentamicin, kanamycin), and first and second generation cephalosporins (cephalexin, cefamandole) [10,189–191].

Suspected cholera should be treated with doxycycline, tetracycline, or erythromycin [10]. Appropriate antibiotic treatment of cholera reduces stool output and duration of diarrhea, which reduces fluid loss, necessity of hospitalization, and shedding of *V. cholera* in stool [181]. Diagnosed, laboratory-confirmed infections should be treated following standard guidelines, but antibiotics should not be used for presumptive treatment. For example, only laboratory-proven, symptomatic infection with *Giardia duodenalis, Entamoeba histolytica*, and amoebiasis should be treated with metronidazole [10,181]. Some have recommended that severe or prolonged diarrhea cases with potential for complications such as sepsis or intravascular coagulation should be treated with antibiotics [191]. In special populations, such as severely malnourished children and children with signs of septic shock, broad spectrum antimicrobials, such as gentamicin and ampicillin, can be given when admitted to a hospital [10].

Antibiotics are contraindicated for the majority of acute diarrhea because: 1) most cases are self-limiting regardless of etiology; 2) antibiotics are not effective against most pathogens associated with diarrhea; 3) antibiotics may have adverse reactions and make the illness worse in the long-term; 4) antibiotics needlessly increase cost of treatment; and 5) indiscriminate use may increase resistance of disease-causing organisms to antibiotics [189,191,192]. Clinically, it is not possible to distinguish diarrhea episodes that might be effectively treated with antibiotics, such as those caused by ETEC, from those that do not respond to antibiotics, such as those caused by rotavirus and *Cryptosporidium* [10]. Even if pathogens are isolated in the stool, they may not be

the causative agent of diarrhea given the substantial asymptomatic prevalence of enteric pathogens in stools of children in LMICs. Antibiotics that are given inappropriately increase the risks for persistent diarrhea and other adverse outcomes, including hemolytic uremic syndrome in EHEC infections and prolonged carriage and shedding of *Salmonella* [53,78,193]. In addition, treatment with antibiotics may reduce focus on, delay, or even replace recommended treatment with ORS, zinc, and continued feeding [189,194].

Treatment with antibiotics is complicated by high prevalence of pathogens resistant to many common antibiotics. Local information on antibiotic sensitivity profiles of the causative microbe is needed to choose the appropriate antibiotic for treatment, but is generally not available [10]. Outbreaks of *Shigella* resistant to more than three antibiotics have been reported in India. Specifically *Shigella* isolates from 2001-2005 in the Indian Subcontinent (India and Bangladesh) were found to be resistant to cotrimoxazole (sulfamethoxazole/trimethoprim; 99% isolates resistant), nalidixic acid (97% isolates resistant), and ciprofloxacin and/or norfloxacin (38.5% isolates resistant). Almost all isolates, however, remained sensitive to cefixime/ceftriaxone. Median percentage of *Shigella* isolates resistant to more than three antimicrobials (multidrug resistant) was 97% across study sites [187]. Similarly, *Vibrio cholera* has been reported to be resistant to cotrimoxazole, ampicillin and furazolidone, though almost all isolates from a clinic in Uttar Pradesh, India in 2006 were sensitive to the recommended treatment with doxycycline [187].

Antibiotic prescribing patterns

International organizations as well as Indian national organizations, including the WHO, UNICEF, the Indian Academy of Paediatrics, and the Ministry of Health, Government of India,

recommend against the routine treatment of diarrhea with antibiotics [10,188,195]. However, antibiotic treatment of diarrhea is widespread in India, mirroring high levels of inappropriate antibiotic use around the world. Multiple surveys of prescribing practices for diarrhea have been undertaken in India to gauge the level of inappropriate use and identify potential strategies for improving rational treatment rates.

The majority of studies have been completed in the hospital setting and report antibiotic prescription rates for acute childhood diarrhea ranging from 0% to 90% [196–203]. For example, among children aged 6 months to 5 years with acute diarrhea (without dysentery, severe malnutrition, or any systemic illness) at an outpatient department of a medical college in West Bengal in 2009-2010, 88.7% and 74.7% were prescribed an antibiotic by a general practitioner and pediatrician respectively—82.5% overall [203]. At a New Delhi medical college in 2005, 53% of outpatient prescriptions by pediatric residents for acute watery diarrhea included antibiotics [199]. Antibiotic prescription rates were similarly high among primary and secondary health care facilities. Among rural and urban government (public) and private facilities at four sites including Vellore, India in 2008, 71% of patients of all ages received an antimicrobial prescription for diarrhea, and 78% of patients with fever and diarrhea were prescribed antimicrobials [204]. Presence of fever was associated with antibiotic prescriptions in several studies; 100% of children aged 1-12 years presenting with fever and diarrhea at a private primary healthcare facility in Chennai in 2005 were prescribed antibiotics [7,198,202,204,205].

Fluoroquinolones were the most commonly reported drugs prescribed for diarrhea across several studies, specifically norfloxacin, ciprofloxacin, and ofloxacin in combination with the antiparasitic, ornidazole [9,201,202,206,207]. Norfloxacin was also commonly combined with metronidazole in a study from Darjeeling, West Bengal [208]. Private providers tend to prescribe

antibiotics more frequently than public providers, and higher medical education is associated with lower antibiotic prescription rates [9,205,207]. In addition, patients of higher socioeconomic status and with more educated mothers are more likely to receive antibiotic prescriptions compared to patients of less-educated and lower socioeconomic status families [7,204,207].

Studies of antibiotic usage in the community are less common than studies of antibiotic prescription rates. NFHS-3 recorded antibiotic usage and reported that among children under 5 who had diarrhea in the two weeks preceding survey, 16% reported treatment with antibiotics and another 30% were treated with unknown drugs [8]. The proportion reporting antibiotic use nationwide had declined from 32% in the first NFHS survey (NFHS-1) [7]. However, use practices varied by state, and the proportion treated with antibiotics in the state of Tamil Nadu (8.5%) during NFHS-3 was half that of the national average [8]. The UNICEF 10-district survey in 2009 estimated that only 5.6% of most recent diarrhea episodes in the 10 surveyed districts (3.8% in Krishnagiri district, Tamil Nadu) had been treated with antibiotics. Service providers reported they gave antibiotics to children if there was blood in the stools, the child was vomiting, or the child showed signs of severe dehydration [43].

Access to antibiotics in India is controlled by the Central Drugs Standard Control Organization. In the Drugs and Cosmetics Act 1940 and Rules 1945 (updated in 2005), antibiotics are classified as “Schedule H” drugs, which cannot be purchased over the counter without the prescription of a registered medical practitioner [209]. However, these regulations are not well-enforced and antibiotics are widely available in pharmacies without a prescription [210]. In the 2011 National Policy for Containment of Antibiotic Resistance, the Government of India acknowledged misuse of antimicrobials. The document includes strategies to establish a

monitoring system and to better enforce current regulations, including a separate schedule, H1, which would have unique provisions applied exclusively to the sale of antibiotics [211]. Despite the ongoing effort to regulate antibiotic use, the drugs are still commonly available in local pharmacies and can be purchased by caregivers without consulting a qualified doctor.

Mothers and other caregivers find antidiarrheal drugs acceptable and desirable since they perceive that treatment with these drugs is effective in stopping diarrhea quickly [210]. Because treatment with ORS solution does not reduce stool volume or duration of diarrhea, caregivers may question its efficacy and turn to other drugs [181]. Physicians often cite parental pressure as a reason for prescribing antibiotics and antibiotic prescription rates are associated with physicians' perceptions of patients' expectations [210,212,213]. Several strategies for reducing inappropriate antibiotic use that address these tendencies have been proposed. A randomized effectiveness trial of zinc supplementation in acute diarrhea determined that antibiotic use was approximately half as prevalent in the group receiving zinc compared to the control group [214]. Zinc decreases the duration of diarrhea and may reduce the incentive for caregivers to give antibiotics when diarrhea persists. In addition, the simple receipt of zinc tablets to treat the diarrhea may satisfy mothers who associate pills with better treatment. A doctor working in a community health clinic in Vellore, India noticed his trend; once doctors at the clinic began giving zinc tablets for diarrhea treatment, the demand for antibiotics decreased [215]. Others have suggested that rapid diagnosis of rotavirus may also reduce inappropriate antibiotic prescribing for diarrhea by providing direct evidence that antibiotics would not be effective for that specific episode [216].

A recent study of the quality of healthcare for childhood diarrhea in rural Bihar, India found a large gap between provider knowledge and practice with respect to antibiotic prescribing

for diarrhea [217]. In structured interviews, the majority of providers (72%) said they would prescribe ORS for uncomplicated diarrhea, and about a quarter (27%) said they would prescribe antibiotics in addition to ORS. Another 21% said they would prescribe antibiotics or other medicines without ORS. In interactions with standardized patients, however, almost all providers (89%) prescribed antibiotics or other harmful drugs, and only 17% of these prescriptions were given in combination with ORS. The large difference between knowledge of appropriate diarrhea treatment (as demonstrated through interviews) and practice (as demonstrated through interactions with standardized patients) suggests other incentives drive antibiotic prescribing practices beyond provider knowledge.

Antibiotic treatment of other childhood illnesses is similarly common around the world, especially for uncomplicated cases of acute gastroenteritis (AGE), upper respiratory infections (URI), and acute otitis media (AOM) [218–220]. Antibiotic treatment is also often unnecessary for these illnesses since most cases are self-limiting regardless of etiology [221,222]. Again, antibiotics are not effective against viral pathogens often responsible for these illnesses [218,223,224], and antibiotics may have adverse reactions or make the illness worse [191,218]. Correspondingly, international organizations, including the World Health Organization, recommend against routine use of antibiotics to treat URI [10,188,218]. Treatment of AOM with antibiotics is more controversial and is recommended for the youngest children. However, deferred antibiotic treatment is often preferred in uncomplicated cases [221,222,225–227]. In the northeastern US, one third of mothers reported that their child received antibiotics before 6 months of age [12]. This prevalence was nearly equivalent to that (32%) reported in a longitudinal birth cohort in the UK [11], though a separate study in Pennsylvania reported only 14% were exposed in early infancy [228]. Given greater access to antibiotics without

prescriptions in low and middle income countries, we would expect similar or higher antibiotic usage rates in India.

Since their discovery in the mid-20th century, antibiotics have quickly become a common exposure among children around the world, even among young infants below 6 months of age. While antibiotic prescription and usage rates vary across patient settings and geographic areas, misuse of antibiotics has been documented across India. Major concerns often focus on the development of pathogen resistance to antibiotics, but direct harm to patients due to inappropriate antibiotic use is also possible and often overlooked [15]. An improved understanding of the effects of this common exposure on short and long-term health is needed.

Effect of antibiotics on diarrhea

Antibiotic-associated diarrhea

Discussions of the impact of antibiotics on diarrheal risk most often focus on short-term effects of antibiotic treatment and the incidence of antibiotic-associated diarrhea (AAD). AAD is a broadly defined disease that is characterized by any diarrhea that cannot be explained by another cause occurring within 8 weeks of exposure to antibiotics [229,230]. Severity of AAD ranges from uncomplicated diarrhea to bloody diarrhea and pseudomembranous colitis [229]. Early onset of diarrhea within 2 to 7 days of antibiotic treatment is common and onset is generally earlier in children than adults. However, delayed onset 2 to 8 weeks after completing antibiotic treatment has also been reported [231].

The prevalence of AAD among patients treated with antibiotics is estimated to range from 5 to 25% depending on the type of antibiotic received, host factors, and hospitalization status. Inpatients generally have higher rates of AAD than outpatients [13,229,230]. Other host

factors that increase risk for AAD are young (<6 years) and old (>65 years) age, underlying disease or comorbid illness, immunosuppression, and history of AAD [13,230,232,233]. Pediatric prevalence of AAD is not well known, and no studies have been completed in India. Estimates from around the world have been 6.2% (Thailand), 11% (US), 16% (Finland), and 17% (Poland) of children who received antibiotics developed AAD. A Cochrane review of trials of probiotics among children 0 to 18 years for pediatric AAD prevention found prevalences of AAD in control groups ranging from 11% to 22% [234]. A study from the United States found highest incidence among kids aged 2 months to 2 years [235].

All antibiotics may potentially be implicated as the cause of AAD, though antibiotics with broad-spectrum activity are most often responsible, especially those targeted against enterobacteria and anaerobic bacteria. Antibiotics with high intraluminal concentration in the intestinal tract, which means that they are poorly absorbed in the upper intestine and reach the colon in high concentrations or are secreted in the intestine through bile ducts, result in the highest risk for AAD [230,236]. Longer duration of antibiotic therapy, including prolonged or repeated therapy, and combination therapies also increase risk for AAD [13,236,237]. Second and third generation cephalosporins, ampicillin, amoxicillin, amoxicillin-clavulanate, clindamycin, and broad-spectrum penicillins have the largest effects on risk of AAD [13,236,238,239]. Fluoroquinolones, tetracyclines, and macrolides have also been implicated in patients with AAD in multiple studies [236,240]. Estimates of AAD prevalences for specific antibiotics include approximately 5-10% of patients treated with ampicillin, 10-25% treated with amoxicillin-clavulanate, 15-20% treated with cefixime, and 2-5% treated with other cephalosporins, fluoroquinolones, clarithromycin, azithromycin, erythromycin and tetracycline [230,239].

The microbes responsible for AAD include *Clostridium difficile, Klebsiella oxytoca, Clostridium perfringens* type A, *Candida albicans* and other *Candida* sp., *Salmonella*, and *Staphylococcus aureus* [13,238,239]. However, no etiologic agent can be identified in approximately 60% of AAD cases, and their etiologies are unknown [229]. Colloquially, *C. difficile* is most often associated with AAD, but the prevalence of *C. difficile* toxin in stool samples from patients with AAD is generally only 10-20% among hospitalized patients [230]. Other researchers estimate that up to one-third of AAD cases are attributable to *C. difficile* [229]. In children, this proportion is estimated to be lower, between 2.5 and 18% [235,241]. In the Indian pediatric population, 3.6% of AAD was associated with *C. difficile* in 1994, and a chart review from an Indian tertiary care hospital in 2008 reported that 6.3% of 60 pediatric AAD cases were associated with *C. difficile*. Studies from Brazil, Europe, and the United States suggest the incidence of *C. difficile*-associated diarrhea acquired in the community may be increasing in children [235,241].

C. difficile-associated diarrhea (CDAD) is generally characterized by more severe diarrhea and is responsible for almost all cases of pseudomembranous colitis [229]. Similar antibiotics have been implicated in CDAD as in non-*C. difficile* associated diarrhea, especially clindamycin, cephalosporins, and penicillins, which are broad spectrum but have little activity against *C. difficile* [230,238,239,242]. Conversely, metronidazole has been associated with decreased risk of CDAD [237]. Initially, fluoroquinolones such as ciprofloxacin were not thought to be a major cause of CDAD given they do not have a large effect on anaerobes in the gastrointestinal tract [230,240]. Newer fluoroquinolones, however, such as moxifloxacin, levofloxacin, and gatifloxacin, have greater activity against anaerobic microorganisms *in vitro* [242]. Correspondingly, more recent reviews, while still showing mixed results, suggest that the

overall evidence demonstrates fluoroquinolones play a role in CDAD, even if not necessarily greater than other broad spectrum antibiotics [242,243].

Longer-term effects of AAD and CDAD have been documented in the recurrence of symptoms [229,235]. Approximately 15-60% of patients with CDAD will experience recurrent disease, which increases length of hospitalization, risk of medical complications, usage of antibiotics, and associated costs. Patients with recurrent CDAD also present with more severe disease [229]. Overall, information on AAD from LMICs is rare, and studies within Indian pediatric populations are needed to understand burden, risk factors, and potential opportunities for prevention [235].

Other effects

Studies of the effects of antibiotics on diarrheal risk outside of AAD and CDAD are rare. However, inappropriate treatment with antibiotics was a risk factor for diarrhea becoming persistent among children below 5 years of age in Pakistan in 1993-1994 [244]. Studies from Bangladesh and India also found that prior antibiotic treatment was associated with persistent diarrhea [245]. In addition, prior antibiotic treatment has been associated with increased susceptibility to *E. coli*, *Salmonella*, *Shigella* and *Campylobacter* infections and with longer duration of infection compared to patients who did not receive antibiotics [246–248]. Antibiotic treatment also reduces the inoculum required to cause infection with *Salmonella* [248]. Supplementation with beneficial bacteria, *Lactobacilli* species, and the yeast, *Saccharomyces boulardii*, have shown the opposite effect of antibiotics by reducing viral shedding and the duration of rotavirus-associated diarrhea [249].

Long-term effects of antibiotics on chronic disease have also been documented, especially for asthma and other allergic diseases. These effects are hypothesized to be due to reduced exposure to microbes, failed development of regulatory immune responses, and promotion of the T-helper type-2 (T_H2) immune response [250,251]. Other immune-mediated pathologies, especially autoimmune disorders, have also been attributed to lack of exposure to microorganisms early in life [251]. Because it could take months or longer for the body to return to a pre-antibiotic exposed state, long-term increased risk for infections after antibiotic treatment is biologically plausible [231]. However, the effects of antibiotics on long-term diarrheal risk are unknown, especially among populations with high incidence of diarrhea such as children in resource-poor settings.

Maternal antibiotic use

Other potential exposures to antibiotics beyond direct administration involve exposure *in utero* and ingestion through breast milk among infants whose mothers are treated with antibiotics. Women who give birth by Cesarean section are commonly given prophylactic antibiotics prior to surgical incision [252]. Women who test positive for Group B *Streptococcus* are also given antibiotics at the beginning of labor to prevent early-onset group B strep disease in their infants [24]. These treatments may expose infants to antibiotic effects before and during delivery. After delivery, antibiotic treatment of mothers may affect their children through breast milk. Recommendations for drug use among women while breastfeeding are based on limited data, but lactating mothers are commonly advised to discontinue breastfeeding while taking antibiotics due to concerns about increased risk of diarrhea in the infant [253–257]. Some

antibiotics are considered to be consistent with breastfeeding, while others are contraindicated among lactating women due to reasons unrelated to diarrhea and are not discussed here.

Almost all (90-99%) breastfeeding women receive medicines within the first week post-delivery [254]. A study of women delivering at the Christian Medical College (CMC) in Vellore, India estimated 37.8% of postpartum mothers were prescribed antibiotics for up to 6 weeks after delivery in 1989. Almost all women having a Cesarean section received antibiotics (96.0%) compared to 35.7% and 20.8% of women receiving antibiotics among instrumental and normal deliveries respectively. Most women received the drugs prophylactically (73.5%), while the remainder of prescriptions were therapeutic. However, 37% of women receiving prophylactic treatment had their prescription extended due to infection or other complication. Cephaloridine was the most common drug prescribed, followed by penicillin/gentamicin combination. These drugs were commonly combined with metronidazole [258]. Similarly, in Chandigarh in 1990, 90% of women who delivered in a tertiary care hospital (45% Cesarean sections) were prescribed antibiotics. However, this proportion was much lower (13%) among women delivering in a community hospital or at home. In this setting, ampicillin was most widely prescribed [259]. Non-compliance among mothers is common given concerns about transferring the antibiotics to infants through breast milk [254].

Estimation of infant exposure to antibiotics through breast milk is difficult to determine given the myriad factors that affect transfer of drugs into breast milk and subsequent absorption in the infant, such as gestational age, time since delivery, maternal factors, inherent characteristics of the drug and drug bioavailability, maternal dosage history, amount of breast milk consumed, and time of antibiotic ingestion relative to infant feeding [254,256,260]. Because most antibiotics are excreted in breast milk, breastfed infants will likely be exposed, but to a dose

much lower than that received by the mother (<1-10%) [254,260]. The clinical relevance of this exposure is not well described, but likely minimal [254]. In addition, because antibiotics are usually prescribed for short periods of time, the infant's exposure is likely to be transient [255,258]. However, newborn infants and infants born prematurely or with comorbidities are at higher risk of adverse events [255].

β-lactam antibiotics (penicillins, ampicillin, amoxicillin), aminoglycosides, tetracyclines, cephalosporins, vancomycin, and nitrofurantoin are found in low concentrations in breast milk, and low drug bioavailability suggests low risk for their use in breastfeeding mothers [254,255,261]. However, the possibility of diarrhea in the breastfeeding infant due to penicillin or cephalosporin exposure has been suggested, though there is no large scale evidence of this phenomenon. Fluoroquinolones, macrolides, sulfonamides, clindamycin, and azithromycin are found in higher concentrations in breast milk, but negligible concentrations are observed in the breastfed infant suggesting the exposure to these antibiotics through breast milk is not clinically relevant. Data on exposure to metronidazole and chloramphenicol is limited and long-term effects on infant health are unknown [254,261], though no adverse effects of metronidazole exposure through breast milk have been reported [257].

One case report described a 2-month-old infant with perforated pseudomembranous colitis after exposure to ciprofloxacin through breast milk, though evidence of causation is weak [254]. Another case report documents bloody diarrhea associated with exposure to gentamicin and clindamycin through breast milk [256]. In the study of prescribing practices in Vellore, one out of 539 infants developed diarrhea while the mother was taking ampicillin [258]. Development of pseudomembranous colitis due to *C. difficile* after exposure to clindamycin through breast milk is a concern, but is expected to be rare. Evidence of diarrhea and rash among

breastfeeding infants associated with penicillins and sulfonamides have also been documented as rare adverse events [255]. While macrolides and, to a small extent, azithromycin can cause diarrhea due to affinity for the motilin receptor, these drug effects have not been consistently documented among breastfed infants [254].

Because a majority of breastfeeding women receive medicines after delivery, better data on infant exposure to drugs through breast milk and resulting health effects are needed. Unnecessary interruption of breastfeeding withholds the many benefits of breastfeeding for the infant and should be advised carefully [254].

Mechanism through the microbiota

The hypothesized mechanism for the effect of antibiotics on diarrhea involves modification of the gastrointestinal (GI) microbiota. The GI microbiota refers to the complex community of microorganisms, including bacteria, archaea, and fungi, inhabiting the human gastrointestinal tract. Approximately 10^{14} microbes live in the GI tract, outnumbering the host's human cells by an order of magnitude and composing up to 60% of fecal matter [20,262]. The total number of genes across the collective species of the microbiota, termed the microbiome, is 2-4 million, which is 100-150 times greater than the number of human genes [18,22,263]. The microbiota has evolved through millennia of host-microorganism interactions resulting in a commensal and symbiotic relationship [21]. The microbiota can be considered a functional organ that plays indispensable roles in the homeostasis of human hosts [22,263,264]. Members of the microbiota are mutualists in that they serve functions for the host and also benefit from the nutrient-rich environment in the host [14,265]. Because the majority of bacteria in the gastrointestinal microbiota cannot be cultured, early studies of the microbiota that relied on

bacterial culture likely provide a skewed representation of microbiota composition. Newer molecular techniques, which most commonly amplify and characterize nucleic acids from the 16S rRNA conserved gene through terminal-restriction fragment length polymorphism, denaturing/temperature gradient gel electrophoresis, and high-throughput sequencing technologies, have allowed much higher resolution in the analyses and closer examination of the complex and diverse communities of the microbiota [266].

Microbiota development

The microbiota develops early in infancy. Exposure to microorganisms as a developing fetus is limited, though the intestinal tract of the fetus during pregnancy is not sterile as previously assumed [21,22,267,268]. Childbirth provides the first major opportunity for microorganisms to colonize the gastrointestinal tract, and mode of delivery has a large effect on the types of organisms that are first introduced to the neonate [269]. For example, infants who are delivered vaginally acquire microorganisms from the mother's vaginal and gastrointestinal flora, especially *Lactobacillus*, *Prevotella*, and *Bifidobacterium*. Conversely, babies delivered by Cesarean section are colonized by organisms common to the skin and non-maternally derived environmental bacteria, such as *Staphylococcus* [21,268,270]. The microbiota of Cesarean section infants is initially less diverse, and intestinal colonization by *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* is delayed [20,21,271].

Hygiene practices during delivery also affect the establishment of the microbiota. Relatively clean deliveries in high-income countries reduce exposure to bacteria and may delay establishment of the microbiota. Infants delivered in LMICs are exposed to a higher bacterial load and have a more diverse microbiota early in life [271–274]. Cesarean section babies from

LMICs acquire bacteria common to the intestine in addition to skin and other environmental bacteria, suggesting fecal contamination of the hospital environment in these settings [274]. Vaginally born and Cesarean section born infants in Pune, India were found to be colonized in high levels by *Acinetobacter* spp. and *C. difficile* respectively [275], suggesting high exposure to potential pathogens in this setting.

Individuals vary greatly in terms of microbial composition, and in contrast to the adult microbiota, the microbiota of infants is unstable and dynamic [23,262,264]. Infants residing in the same geographic area had microbiotas with wide variation in composition over the first year of life. However, the similarities between microbiotas of twins suggest common genetics and/or environmental exposures contribute to the distinctive characteristics of microbiotas across infants [276]. The greatest changes in composition of the microbiota occurs during a process of bacterial succession throughout infancy, in which diversity increases with time [267,277]. Initially, the gastrointestinal environment in the infant is aerobic and encourages colonization of aerobes and facultative anaerobes, including enterobacteria and Firmicutes, specifically *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Lactobacillus* [20,271,278,279]. However, early colonizers reduce oxygen levels, which results in the growth of obligate anaerobes, such as *Bifidobacterium*, *Clostridium*, and *Bacteroides* [262,268,271,278,280]. The weaning process, with the introduction of solid foods, induces a major change in the microbiota [281,282]. The microbiota starts to resemble that in the adult gastrointestinal tract by 1 year of age and almost completely by 3 years of age [21,23,262,268,277,283]. However, at 2 years of age, facultative anaerobes are more often found in children compared to healthy adults, and complete resemblance to the adult microbiota in level of diversity does not occur until later in childhood

[268,277]. Differences in microbiota composition soon after birth could have effects in the intestine for up to 7 years [21].

Other factors influencing microbiota composition early in life include genetic factors, geography, breastfeeding and diet, health and microbiota of the mother, gestational age, family structure, and exposure to antibiotics [21,23,262,264,268,279–281,283–286]. Preterm infants have immature gastrointestinal tracts and colonization of the intestine is delayed, resulting in lower diversity of the microbiota [23,263,284]. The differences in microbiota composition of breastfed infants compared to older children (no longer exclusively breastfed) and adults were initially described using culture techniques in the late 1960s [287]. Oligosaccharides abundant in breast milk are unable to be fully digested by infants and instead are consumed by and enrich the microbiota, especially *Bifidobacterium* [288–290]. Correspondingly, the predominant bacteria in the microbiota of breastfed infants are *Bifidobacterium* and *Lactobacillus* [22,277], while infants fed with formula milk have a more complex microbiota that is more similar to the microbiota of adults [23,270,271,277]. Taken together, the determinants of the microbiota suggest full-term infants born vaginally and who are exclusively breastfed have the "most beneficial" microbiota composition, with many *Bifidobacterium* and fewer *E. coli* and *C. difficile* [23,280,291].

Diet following the cessation of breastfeeding is also an important determinant of microbiota composition. Diets high in plant carbohydrates favor colonization by bacteria that are able to ferment dietary fiber [292]. Children from Burkina Faso with a high-fiber diet showed increases in Bacteroidetes and decreases in Firmicutes in comparison to European children with a modern western diet. An abundance of *Prevotella* and *Xylanibacter*, which contain genes that ferment cellulose and xylan, in the children from Burkina Faso suggests their high-fiber diet may have influenced the composition of their microbiotas to maximize energy intake from ingested

food [293]. It is likely that only long-term changes in diet (over months or years) are able to substantially shift the composition of the microbiota [294].

While the microbiota of infants are individualized and dynamic, the microbiota in adults is more stable and less modifiable by exposures and life events [295]. However, microbial populations are highly variable across populations and within populations over time [296]. The main bacterial phyla of the adult gastrointestinal microbiota are Bacteroidetes and Firmicutes, comprising over 90% of the total microorganisms [20,279,297,298]. Minor constituents include Actinobacteria (specifically *Bifidobacterium*), Proteobacteria, and Verrucomicrobia bacteria as well as methanogenic archaea, eukaryotes (mostly yeasts), and viruses/phages [296,298]. Most of the bacteria in the adult intestine are strict anaerobes, with aerobic and facultative anaerobes at much lower prevalences [272]. Adult human intestinal microbiota can be classified into three enterotypes which describe the three observed patterns of dominating taxa: *Bacteroides* in enterotype 1, *Prevotella* in enterotype 2, and *Ruminococcus* in enterotype 3. Prevalence of enterotypes is highly associated with diet; for example, diets heavy in protein and animal fat are associated with enterotype 1, while diets composed of mostly carbohydrates are associated with enterotype 2 [264]. However, other factors such as age, gender, nationality, and body mass index are not associated with enterotype. While the composition of organisms is different between enterotypes, they form similar functions and each create homeostasis [270]. Some speculate that the classification of enterotypes is artificial and they are not distinct, but instead lie on a continuum. Corresponding enterotypes among children have not yet been described [20].

Microbiota function

The importance of the microbiota in human health was first postulated by Élie Metchnikoff in 1907, who thought certain gut bacteria poisoned the body. He associated longer lifespan with consumption of fermented milk containing beneficial lactic acid bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophiles*) [281,299,300]. In his book, *The Prolongation of Life*, he writes, "I think, therefore, that lactic bacteria can render a great service in the fight against intestinal putrefaction" [300].

Research following Metchnikoff's observations has demonstrated that the microbiota is important during early development for intestinal structure, metabolism, nutrition, and normal immune system development [21]. The functionality of the microbiota is encoded in approximately 20,000 microbial genes, a third of which are well-characterized [301]. While microbiota composition in terms of bacteria species can vary greatly, taxonomic diversity is not correlated with functional diversity, and the functions performed by the microbiota are similar across individuals suggesting there is functional redundancy across bacteria [296,301]. In addition, bacterial species exist in an interrelated network and rely on other bacteria for complementary functions which leads to correlated fluctuations in the abundances of functionally related species [301].

Most conclusions about the function of the microbiota are derived from studies of germ-free mice, which have no intestinal microbes and can be functionally compared to normal mice [279]. The microbiota plays a role in developing the normal intestinal layer by affecting gene expression associated with angiogenesis and maturation of the intestine [22]. Germ-free mice do not fully develop intestinal blood vessels, and villus capillaries remain underdeveloped through adulthood [279,302]. In addition, germ-free mice have intestinal structural abnormalities

including enlarged cecum, dysfunctional gut-associated lymphoid tissue (GALT), and reduced intestinal surface area, epithelial cell turnover, and Peyer's Patches [22,303]. The proliferation and differentiation of epithelial cells as well as the maturation of the intestinal mucosa and GALT requires signaling from the intestinal microbiota for complete development and recruitment of mature immune cells [265,301]. The microbiota therefore plays a role in intestinal barrier function, and aberrant microbial colonization early in life may increase permeability of the intestine and mucosal inflammation [22].

The microbiota also supports normal digestion and metabolic functions by affecting nutrient absorption and energy storage in the host [22,23]. A large number of novel genes are found in the microbiota which supplement the limited enzymes encoded in the human genome that metabolize complex carbohydrates and proteins [265,304]. For example, bacteria ferment remaining energy substrates from ingested foods to short chain fatty acids, breakdown proteins into their essential amino acids, and facilitate the extraction and storage of calories into host fat tissue [22,23]. Children from Burkina Faso were found to have more Bacteroidetes compared to European children, corresponding to more short-chain fatty acids, which provide beneficial anti-inflammatory functions [293,305]. The microbiota also provides and metabolizes vitamins and other non-nutrient factors which are essential for human health [265,306]. Finally, the microbiota contributes to healthy sensory and motor gut functions by contributing to intestinal propulsive activity [302] and through the brain-gut- microbiota axis, which allows the microbiota to interact with the brain through neuronal cells and epithelial-cell and receptor-mediated signaling [22,267]. Recent evidence even suggests the microbiota plays a role in brain development and can subsequently influence adult behavior [301].

The changes and development of the microbiota in the first few years of life coincide with a critical period of immune system maturation, such that the microbiota contributes to developing the immune system, and the composition of the microbiota and its access to body sites is reciprocally controlled by the immune system [279,303]. The microbiota contributes to the balance of T cell subsets, including regulatory T (T_{reg}) cells and those involved T_H1 and T_H2 type immune responses, which influences recognition of microbes by gut immune cells to initiate appropriate immune responses [22,303]. Deviations in microbiota composition have been associated with allergic and autoimmune diseases, such as inflammatory bowel disease (IBD), type 1 diabetes mellitus, and asthma, which are associated with pathological T_H1 and T_H2 responses [23]. By stimulating lymphoid tissue in the gut mucosa, the commensal bacteria direct the immune system to recognize and produce antibodies (especially secretory IgA) against pathogens while not harming helpful bacteria, a process termed immune tolerance. This activity is mediated by the production of T_{reg} cells and expression of toll-like (pattern recognition) receptors in the intestines which discriminate between commensal bacteria and pathogens [23,246,269,279,305,307]. In addition, the microbiota aids in the development of oral tolerance, in which the immune system does not respond to ingested food or self-antigens [22,269]. Germ-free mice show reduced IgA antibody concentrations and lower concentrations of circulating B and T lymphocytes compared to normal mice, and correspondingly respond to infection and injury with ineffective immune responses [246,265,279].

The microbiota and diarrhea

The first hypothesis that the intestinal flora protects against infection was proposed in 1916 by the German scientist Alfred Nissle, and supporting evidence for this hypothesis

continues to grow [308]. In addition to the beneficial effects of the microbiota on immune system homeostasis described above, a healthy microbiota may protect against diarrheal disease through a barrier effect called competitive exclusion, such that members of the microbiota occupy intestinal mucosal sites which inhibits the attachment and growth of pathogens [14,299,301,309,310]. This process has also been referred to as colonization resistance or a barrier effect, in which the normal microbes are a barrier against colonization of pathogens and overgrowth of yeasts [302,308,311]. Commensal bacteria also discourage growth of pathogens by competing for nutrients, directly releasing inhibitory molecules, and impairing flagellar motility [14,302,308,309]. Because resident microorganisms are well-adopted to the intestinal environment, generally inhabit available metabolic and physical niches, and have established robust networks through biofilms, healthy microbiotas resist the establishment of pathogens that might cause diarrhea [265].

The role of the microbiota in affecting susceptibility to infection has been well-studied in mouse models, and early studies from the 1960s and 1970s showed the intestinal flora was antagonistic against *Salmonella, Shigella,* and *Vibrio cholera* infection [308]. Several other recent studies have found that a normal microbiota in mice successfully prevents colonization by *Salmonella enterica* serovar Typhimurium, likely through competitive exclusion. Conversely, mice with altered microbiotas due to antibiotic administration are more susceptible to intestinal infection and disease due to *Salmonella* and other enterobacteria such as *E. coli* [247,249,312]. One study showed a dose-response such that greater alterations to the microbiota led to higher colonization by *S. enterica* serovar Typhimurium, and more severe inflammation and intestinal pathology. Because the microbiota-modified mice did not have reduced total bacterial numbers, the alteration in bacterial composition appears to be responsible for the increased susceptibility

[312,313]. Further, modification of the microbiota through the antibiotic treatment of mice increased susceptibility to infection by vancomycin-resistant *Enterococcus* and *C. difficile* [312]. Increased susceptibility to pathogens may be partially due to reduced host-produced antimicrobial molecules when the microbiota is disrupted [247]. The clear association between the microbiota and susceptibility to infection has led some researchers to suggest that people with an altered microbiota are functionally immunocompromised and less resilient against new and opportunistic pathogens and recurrent infections [312].

The association between the microbiota and susceptibility to viral infection and disease is not as well-understood. Studies have found examples both where intestinal bacteria are antagonistic to viral infection and where they promote viral infection [247,314,315]. For example, *Bacteroides thetaiotaomicron* and *Lactobacillus casei* have been shown to prevent infection of the intestinal epithelial cells by rotavirus *in vitro*. Similarly, mice with depleted microbiotas through antibiotic treatment or development in germ-free conditions are more susceptible to influenza compared to normal mice [247,314]. In this case, the microbiota appears to initiate, and may be required, for the immune response against influenza since mice with altered microbiotas showed reduced antibody titers and T cell responses [314,315].

On the other hand, the GI microbiota has been shown to enhance replication and infection of other viruses [314,316,317]. Antibiotic-treated mice were less susceptible to poliovirus compared to mice with normal microbiotas, resulting in a mortality rate among normal mice twice that among antibiotic-treated mice. When bacteria were reintroduced to the antibiotic-treated mice, the pathogenesis of poliovirus increased. Similarly, the pathogenesis of reovirus (another enteric virus) was enhanced in mice with normal microbiotas. The authors conclude that antibiotic-disruption of the microbiota may have anti-viral effects despite no direct action against

viruses [316]. A similar study demonstrated that mouse mammary tumor virus, a retrovirus, was more efficiently transmitted in the presence of a rich microbiota, and correspondingly virus transmission to offspring was reduced in antibiotic-treated mice and germ-free mice [317]. *In vitro* studies in mouse and human cell lines confirm that components of both Gram-positive and Gram-negative bacteria increase the infectivity of viruses [315].

Further, viral infections can enhance secondary bacterial infections suggesting a close interconnected relationship between the microbiota, bacteria, and viruses [314]. Astroviruses and rotaviruses increase the permeability of the gut mucosa, which compromises the immune response to a wide range of pathogens [249]. Recent evidence suggests that other disorders associated with the microbiota, such as IBD and Crohn's disease, are mediated not only by the commensal bacteria, but also by enteric viruses [249]. Finally, the role of the microbiota in resistance to fungal infections has been demonstrated by the association of antibiotic treatment with fungal infections by *Candida albicans* [247,308].

The potential for dysbiosis of the microbiota to specifically cause acute infectious diarrhea in humans, in addition to infection more generally, has been less well-studied. Several researchers have documented changes in the microbiota during diarrhea. Most studies are cross-sectional and compare the microbiotas of diarrhea cases and healthy controls. These studies excluded subjects who had recently taken antibiotics to ensure changes in the microbiota were associated with diarrhea alone. The first studies were conducted in the 1970s and 1980s and relied on bacterial culture to describe microbiota composition. Mata, in 1972, found that in the neonatal period, the microbiotas of children with severe diarrhea with dehydration showed a substantial decrease in anaerobes, especially *Bifidobacterium* and *Bacteroides*. *E. coli* and other enterobacteria comprised the majority of the flora, and *Shigella* was found in high numbers

[318]. Two studies from Vellore, India in the 1970s similarly documented that the proportion of aerobic to anaerobic bacteria was unusually high in diarrhea cases. Subjects with diarrhea had more enterobacteria and *Staphylococcus*, while healthy controls had more *Bifidobacterium*, *Bacteroides*, and V*eillonella* [319,320]. These studies suggest diarrhea may induce a more aerobic environment in the gastrointestinal tract, which promotes growth of aerobes over anaerobes.

Since the advent of highly sophisticated bacterial DNA detection techniques, researchers have been able to identify large numbers of organisms in the microbiota that could not be readily cultured in earlier studies. These techniques have implicated the dysbiosis of the microbiome in many chronic gastrointestinal diseases in humans including enteric infections, especially *C. difficile* infection, small intestinal bacterial overgrowth, inflammatory gastrointestinal disorders such as irritable bowel syndrome (IBS) and IBD, and colorectal cancer [246]. DNA sequencing of the 16S rRNA gene (highly conserved across bacterial species) in fecal samples from patients with diarrhea-predominant IBS showed the microbiotas of IBS patients were less diverse and more instable over time compared to healthy controls [321,322]. Specifically, IBS patients had more enterobacteria, *Veillonella*, *Prevotella*, and *Lactobacillus*, and less *Faecalibacterium*, *Bifidobacterium*, and Verrucomicrobia compared to controls [321,323]. Similarly, patients with *C. difficile*-associated diarrhea (CDAD) and infants with *C. difficile* colonization had reduced diversity and high variability in fecal bacterial communities. Patients with *C. difficile* infection also showed reduced Firmicutes and Bacteroidetes populations [246]. Again, the ratio of facultative anaerobes to strict anaerobes was higher in patients with CDAD, and prevalence of *Bifidobacterium* was inversely associated with *C. difficile* [324–328]. Among a small sample of

men with diarrhea induced by an osmotic laxative, microbiota diversity was reduced and a shift in prevalent phyla was documented from Bacteroidetes and Firmicutes to Proteobacteria [329].

Variations in the microbiota associated with acute (presumably infectious) diarrhea have also been demonstrated using molecular techniques in both high and low-income country settings. Diarrhea patients presenting to a hospital or clinic in the United States showed decreased diversity and overgrowth of selected organisms in fecal samples compared to healthy clinic controls [330]. Among children in Bangladesh, acute diarrhea was associated with decreased microbiota diversity, and cholera patients had reductions in Bacteroidetes, Firmicutes, and Actinobacteria, with an increase in harmful Proteobacteria [331,332]. Similarly, Colombian children who had diarrhea in the past 2 weeks had a reduced copy number for total bacteria in fecal samples and fewer *Bifidobacterium* and L*actobacillus*. However, relative concentrations of bacterial species in diarrheal fecal samples varied across study sites [333]. The microbiotas of adult patients in China with viral diarrheas caused by adenovirus, norovirus, rotavirus, and astrovirus were less diverse and more variable compared to healthy controls. The dominant phylum in diarrhea cases was Firmicutes instead of Bacteroidetes, and *Bacteroides, Bifidobacterium,* and *Lactobacillus* were found in lower copy numbers among patients with diarrhea [334]. Children aged 3 months to 5 years with acute diarrhea and mild dehydration in Vellore, India had lower levels of *Bacteroides-Prevotella* group bacteria during diarrhea compared to 3 months after diarrhea, while no disturbance of *Bifidobacterium* was observed [335]. The same researchers showed asymptomatic rotavirus in neonates in the first month of life did not alter *Bifidobacterium* or enterobacteria counts in stool samples [336].

These studies have consistently documented changes in the microbiota during diarrhea, but do not establish temporality. It is not clear from fecal samples collected during the diarrhea

episode if diarrhea causes the modifications in the microbiome, if dysbiosis of the microbiome instead is a risk factor for diarrhea, or if both processes are possible. However, the evidence described above from animal models suggest dysbiosis of the microbiota increases susceptibility to infection and could therefore be an important risk factor for diarrhea.

The role of the microbiota in diarrhea, susceptibility to infection, and other GI disorders is also supported by the success of fecal microbiota transplantation (FMT) in ameliorating GI disease. FMT involves introducing an entire microbial community to a patient through administration of a healthy donor fecal sample by enema, transcolonic infusion, or nasoduodenal or nasogastric infusion. The goal of FMT is to replace an unhealthy microbiota with a healthy one, and has been associated with increases in richness and diversity of the microbiota [328,337]. Few adverse events have been reported, and FMT has shown to be effective most commonly in treating *C. difficile* infection, but also in treating ulcerative colitis and IBS [337,338]. Supplementation of the microbiota with the probiotics, *Bifidobacterium bifidum* and *Streptococcus thermophilus*, in infant formula also resulted in reduced diarrhea incidence in a small study of children under 2 years of age [339]. The ability for a supplemented or replaced microbiota to improve GI disorders indicates the role of the microbiota in gastrointestinal pathogenesis.

Antibiotics and the microbiota

Antibiotic treatment is a major cause of disturbances to the microbiota that may induce diarrhea and predispose to other diseases. Because many commonly-used antibiotics target a broad range of bacteria, antibiotics are effective in killing not only pathogenic bacteria, but also beneficial commensal microorganisms in the gut [18]. By targeting a subset of the bacteria in the

microbiota based on drug activity, antibiotic treatment affects the relative abundance of organisms in addition to their absolute numbers. The substantial reduction of beneficial bacterial populations provides the opportunity for overgrowth of opportunistic pathogens and increase in disease severity [14,340]. Specifically, drug resistant bacteria are able to flourish under antibiotic selective pressure while sensitive bacteria are depleted [17,292,341,341]. For example, *C. difficile* is often found in low prevalences and is non-pathogenic until the normal flora is depleted by antibiotics and *C. difficile* is able to occupy newly available ecological niches [17,340]. Further, even if not directly targeted by the antibiotic, bacteria may be depleted due to dependences on targeted bacteria for nutrients, secondary metabolites, or waste product removal. For example, treatment with vancomycin reduced the abundance of Gram-negative organisms despite the restriction of antibiotic activity to Gram-positive bacteria [14,19].

The collateral damage from antibiotics to the healthy microbiota has been repeatedly shown to cause dramatic short-term changes to microbiota composition, wherein reduces in microbial diversity occur in the first few days of antibiotic exposure. Bacteria resistant to the antibiotic increase in numbers and dominate the microbiota until antibiotic pressure is removed and sensitive bacteria are found again in increased numbers. However, antibiotics can also cause lasting effects such that the microbiota does not fully recover to its pre-treatment state [18,20]. The magnitude and type of changes induced in the microbiota depends on the spectrum of bacteria covered by the antibiotic, the dosage, duration, and route of administration of treatment, and the pharmacokinetic and pharmacodynamic properties of the antibiotic [17,20,247,311].

While many studies of the effects of antibiotics on the microbiota have been completed in mice and other animal models, studies of the effects of antibiotics in humans have been more unusual. These studies often involve a small number of subjects and are complicated by baseline

variability in microbiota composition [248,286]. Because responses to antibiotics are individualized (large among-subject variability) and are influenced by prior exposure to antibiotics, aggregation of microbiota composition data across subjects may not produce valid results. Comparison of samples taken from the same individual before and after treatment are likely to be more interpretable [17,286,340]. Further, studies among sick patients with clinical indications for antibiotic treatment are confounded by effects the indicating illness may have on the microbiota [286]. However, results from human studies are consistent in that in nearly all studies across specific antibiotic exposures, antibiotics caused a sharp reduction in the abundance and diversity of organisms in the microbiota [17,18,292]. Antibiotic use has also been repeatedly associated with reductions in Firmicutes and Bacteroidetes and a concurrent increase in Proteobacteria [18].

Because the majority of bacteria in the human gastrointestinal tract are anaerobic, antibiotics that are active against anaerobic bacteria, such as clindamycin, may have the largest effects on the microbiota and normal GI functioning [17]. Broad spectrum antibiotics also have a larger impact compared to narrow-spectrum antibiotics active against few bacteria [251]. Because the microbiota of infants and young children is underdeveloped, relatively unstable, and highly susceptible to disturbances, antibiotic exposures early in life may delay normal intestinal colonization and have the largest and longest-term effects on the microbiota. Specifically, microbiota modifications are pronounced among infants under 1 year of age, and changes to microbiota composition last longer in neonates exposed to antibiotics compared to 10-month old exposed infants [20,251,280,291,340].

Antibiotic use in infants has been associated with decreased numbers of *Bifidobacterium* and *Bacteroides*, and increased numbers of *Clostridium, Enterococcus, Staphylococcus*, and

enterobacteria in stool samples [19,247,268,280,283,297,340,342]. For example, one week of amoxicillin treatment for acute bronchitis among infants aged 1-2 years resulted in decreased total fecal bacteria and increased abundance of *E. coli* [343]. Antibiotic treatment of over 600 European infants in the first 6 weeks of life was associated with higher relative proportions of enterobacteria (16.6% of total bacteria in infants treated with antibiotics versus 6.8% in untreated infants) [344]. Similarly, neonates given parenteral ampicillin and gentamicin with 48 hours of birth had more Proteobacteria and less Actinobacteria, including *Bifidobacterium* and *Lactobacillus*, than untreated neonates 4 weeks after treatment [345].

The evidence concerning long-term effects of antibiotics on the microbiota is mixed. The response of the microbiota over time to disturbances due to antibiotic exposure has been studied within an ecological framework, specifically assessing ecosystem stability and resilience [286,346]. The complexity of the microbiota community and the functional redundancy therein may contribute to the long-term resiliency of the microbiota in response to disturbances by antibiotics or other interventions [301]. Correspondingly, studies demonstrate that the majority of bacterial species return to their pre-treatment abundances relatively quickly. However, some species may not recolonize for an extended period of time (> 4 years) or not at all. Therefore, the recovery of the microbiota following antibiotic exposure is often incomplete [16,296,297,341,347–353]. For example, the composition of the microbiota of healthy volunteers almost fully returned to its pre-treatment abundances 4 weeks after oral treatment with ciprofloxacin for 5 days, but some bacterial taxa did not recover at 6 months post-treatment [16]. Similarly, alterations of some species in the microbiota persisted for up to 2 years following treatment with clindamycin for 7 days [292,352] and remained for up to 4 years after treatment with clarithromycin, metronidazole, and omeprazole for *Helicobacter pylori* [354]. In infants,

overgrowth of enterobacteria after antibiotic treatment persisted to at least 1 month after treatment with cephalexin in the first 4 days of life [342]. Similarly, higher levels of Proteobacteria and reduced diversity of *Bifidobacterium* species due to parenteral treatment with ampicillin and gentamicin within 48 hours of birth persisted at 8 weeks of life [345].

In addition to affecting the types and numbers of bacteria in the gastrointestinal tract, antibiotics further alter metabolic activities, vitamin absorption, and immune system development and functioning in the gut [17,251,280,355]. Antibiotic use resulted in altered amounts of metabolites found in mouse fecal samples, suggesting antibiotics affect pathways associated with sugar, nucleotide, and fatty acid metabolism in addition to bile acid, eicosanoid, and steroid hormone synthesis [14,356]. Alterations to the microbiota may also change our ability to metabolize drugs, resulting in differences in activation or inactivation, prolonged circulation, and increased toxicity of drugs [340,355]. Antibiotic exposure in the perinatal period has been shown to result in changes in gene expression associated with the developing GI tract, which may result in impaired GI functioning, intestinal inflammation, increased intestinal permeability, and increased risk of systemic infections [301,340,357]. Loss of bacterial signals and bacterial components that are recognized by the immune system impacts inflammatory and other immune responses, especially the development of regulatory lymphocytes [14,251]. Mice treated with antibiotics have shown reduced lymphoid tissue, neutrophil activity, T_H1 responses, and interferon, cytokine, and IgG serum levels [19,251]. Antibiotics may even inhibit the development of protective responses after exposure to vaccines, while probiotics that enhance the microbiota may increase the immunogenicity of vaccines [315,358]. Finally, by selecting for resistant bacteria, antibiotic treatment increases the reservoir of resistance genes present in the

microbiota that could be transferred between species, potentially reducing the effectiveness of future antibiotic treatment in the individual [14,248,341,359].

Maternal antibiotic use and the infant microbiota

The hypothesized mechanism for the effect of maternal antibiotic use on infant diarrhea also involves modification of the infant microbiota due to exposure to antibiotics in breast milk. Maternal antibiotic use during the perinatal period alters the developing microbiota in the neonate and may cause overgrowth of potential pathogens [251]. Changes in the microbiota may also mediate the effect of perinatal exposure to antibiotics on increased risk of necrotizing enterocolitis, cerebral palsy, and IBD [23]. The microbial diversity of infant stool samples was reduced in infants of mothers who were given antibiotics soon before delivery in one study [340], and antibiotic treatment of mothers prenatally or during breastfeeding was associated with lower total numbers of bacteria and lower proportions of *Bacteroides* and *Atopobium* in another [268,344]. However, the effects of antibiotic exposure in the infant due to treatment of the mother are likely to be weaker compared to direct antibiotic exposure. Cesarean section babies with mothers who were treated intravenously with broad-spectrum cefotiam hydrochlorlide in the first 4 days of life had similar types of alterations to the microbiota as babies directly administered antibiotics in terms of reductions in diversity and *Bifidobacterium* and overgrowth of *Enterococcus*, but the alterations were less pronounced [342]. Several studies have found antibiotic use during pregnancy has no effect on the infant microbiota [268,297].

In sum, the evidence for substantial effects of antibiotic exposure on the microbiota, and the corresponding association between microbiota dysbiosis and increased susceptibility to infection, suggest a highly plausible mechanism for an effect of antibiotics on diarrheal risk.

However, the contribution of antibiotic treatment to diarrheal risk in young children in LMICs is unknown.

Antibiotics, the microbiota, and growth

Antibiotics may indirectly affect growth by increasing the number, duration, and severity of diarrhea episodes, which would in turn increase the risk for growth shortfalls as described previously. However, antibiotics may also have a more direct effect on malnutrition and growth outcomes mediated by the changes in the microbiota. The hypothesis that antibiotics and the microbiota may affect growth originated in the food animal industry, where antibiotics are administered to animals at low doses for an extended period time in drinking water and commercial feeds [74,360,361]. The ability for antibiotics to promote growth in livestock has been documented since the 1950s. Antibiotic use for growth promotion increases the rates of weight gain, especially in poultry and swine, by up to 16% [74,360]. Although the specific mechanism is unknown, modification of the microbiota by antibiotics and alteration of the animals' immune responses likely play a role in the growth promoting effect. Antibiotics have been shown to influence the diversity of the microbiota in chickens [362], and do not promote growth in germ-free animals, suggesting the microbiota is a necessary mediator of this phenomenon [361]. Because a variety of antibiotics increase growth, including macrolides, tetracyclines, penicillins, and glycopeptide, the effects do not appear to be specific to a certain drug class or type [360,363,364].

Analogous treatment to increase weight gain in malnourished children with long-term daily administration of antibiotics produced conflicting results in studies during the 1950s. Severely undernourished African children given aureomycin for 2-7 weeks had higher weight

gains than children given a placebo. Similarly, Guatemalan children fed 50 mg of aureomycin daily for 6 months grew larger in weight and height compared to children given placebos. However, there was no long-term height and weight advantage at 2 years after treatment, and penicillin had no effects on either height or weight gain. The authors of a review of these studies conclude that there was no evidence that prolonged treatment with antibiotics increased growth in children [74].

However, short-term courses of antibiotics are widely used to treat acute malnutrition, and the WHO recommends that all severely malnourished children receive broad-spectrum antibiotics, such as gentamicin and ampicillin, for several days if admitted to a hospital [10]. Antibiotic use in this setting is thought to treat or prevent disease which allows children to regain weight as they recover. A study of malnourished Guatemalan children in 1972 found that children with protein-calorie malnutrition had more enterobacteria in the small intestine and an altered fecal flora compared to normal children [365]. Lack of dietary protein was linked to overgrowth of intestinal bacteria in the guts of children with kwashiorkor [366], and differences were found in microbiota composition between twins discordant for kwashiorkor [367]. Similarly, analysis of the microbiota from a malnourished child from an urban slum in Kolkata showed evidence of infection by gastrointestinal pathogens belonging to the *Campylobacteraceae* and *Helicobacteraceae* families, which may respond to antibiotics [368]. Correspondingly, amoxicillin and cefdinir have been associated with increased weight gain in undernourished Malawian children [360]. In a randomized trial of 7-day courses of amoxicillin or cefdinir for severe acute malnutrition among Malawian children under 5 years of age, recovery rates were 3.6-5.8% higher and mortality rates were 2.6-3.3% lower among children receiving antibiotics. The rate of weight gain was also faster among children receiving antibiotics

[369]. Antibiotic treatment likely affects the microbiota, which could contribute to the pathogenesis and recovery from undernutrition, through its impact on both nutrient metabolism and immune system functioning [370].

Antibiotics have also been associated with weight gain in children without malnutrition. Erythromycin increased daily weight gain in preterm infants with feeding intolerance [371]. Administration of sulfonamides and cotrimoxazole to prevent pneumonia and other complications after measles also increased weight gain among children in Guinea-Bissau [372]. Several other studies have linked tetracyclines, macrolides (especially azithromycin), and clarithromycin to weight gain in infants, older children, and adults [363]. Antibiotics in different settings may contribute to weight gain by preventing or treating infection and by causing changes in the composition of the microbiota, or both [363].

Antibiotics given to infants in the first 6 months of life may have the largest effects on growth given antibiotic use at this age has been associated with being overweight later in childhood, while antibiotic use at 6-23 months was not shown to impact later growth [11,360,363]. However, this association is likely more nuanced, as antibiotics during the first 6 months of life increased risk of overweight among Danish children of normal weight mothers, while it decreased the risk of overweight among Danish children of overweight mothers [373]. In a trial of annual versus biannual mass oral azithromycin distributions for trachoma in Niger, no significant difference in anthropometric measurements of preschool children were found, though biannually treated children had slightly lower odds of underweight, stunting, and wasting [374].

A recent meta-analysis of 10 randomized control trials of oral antibiotics in low or middle income countries concluded that antibiotics improved growth, though the summary effect sizes were likely not clinically significant (less than 1 mm/month difference in height and 24 g/month

in weight) [375]. These trials included the early studies mentioned above and therefore were conducted over a 60 year period and varied broadly in terms of indication for treatment, eligibility, and antibiotic intervention. An international cross-sectional study of antibiotic exposures in the first year of life also reported an adjusted increase in body mass index (BMI) associated with antibiotics at age 5-8, but only among males (+0.104 kg/m^2). The effects varied across sites and a decrease in BMI was found in all countries classified as non-affluent except Thailand [376].

The impact of antibiotics and the microbiota on growth, and specifically on weight gain and loss, has recently garnered renewed interest in light of the growing problem of obesity. Because the functional repertoire of the microbiota includes energy harvest and fat deposition, different compositions of the microbiota may be more efficient in energy uptake than others and therefore contribute to excessive weight gain in humans [377–379]. Recent studies have shown consistent differences in the microbiotas between lean and obese mice, specifically a shift in the ratio of Firmicutes:Bacteroidetes with a higher than normal abundance of Firmicutes in obese mice [363,380,381]. Germ-free mice have lower body fat content than normally raised mice even when the germ-free mice consume more food [382]. The body fat content of germ-free mice when colonized with the microbiota from a conventionally raised mouse increases by 60% within two weeks, even with reduced food intake [383]. When germ-free mice were colonized with the microbiotas from obese mice, they showed higher weight gain compared to germ-free mice colonized with microbiotas from lean mice. These mice had higher abundances of Firmicutes and correspondingly had increased energy extraction from food and up regulation of genes in the microbiome involved with carbohydrate and lipid metabolism [363,379]. Conversely, when the microbiotas from human infants with kwashiorkor were transplanted into

germ-free mice and the mice were given a diet similar to the infants' diet, these mice lost significant weight, mirroring the phenotype of kwashiorkor [367]. These studies suggests that the microbiota in interaction with diet was responsible for the overweight and underweight phenotypes respectively.

Because there are more genes involved in lipid and carbohydrate metabolism in Firmicutes compared to Bacteroidetes, researchers speculate that Firmicutes may contribute to greater energy harvest [382,384]. This hypothesis has been supported by concurrent increases in Firmicutes, increases in weight gain, and alterations of carbohydrate, lipid, and cholesterol metabolism, including an increase in fatty acids, in the guts of mice treated with antibiotics [382]. However, differences in metabolic function at the family and species levels indicate that there is not a uniform separation among species within the two phyla [363]. The Firmicutes:Bacteroidetes ratio may be an oversimplification and is likely modified by diet [380].

This conclusion is supported by human studies wherein the Firmicutes:Bacteroidetes ratio has been shown to be both increased and decreased among obese humans in different settings [363,380]. Among healthy adults in India, there was no clear association between the Firmicutes:Bacteroidetes ratio and obesity [385]. Actinobacteria, *Lactobacillus* species, and several other bacteria species have also been associated with obesity in different studies [363,381,386]. Among infants, higher levels of *Bacteroides* the first year of life was associated higher body mass index in the 2nd and 3rd years of life, taking into account several important risk factors for body mass index. In another study, increased body mass index associated with microbiota composition differences in the first year of life persisted to affect risk of overweight at 7 years of age [379].

These studies indicate large variation in the effects of specific bacterial species on weight gain, and some of these differences are likely due to methodological challenges. Cross-sectional studies prohibit the conclusion of a temporal relationship between microbiota composition and obesity, while observational longitudinal studies may be confounded by common risk factors for the microbiota and growth. In addition, sampling of the microbiota from the large intestine or in fecal samples may be misleading since most metabolic activities associated with the microbiota occur in the small intestine [361].

Recent epidemiologic studies from high-income countries have reported associations between antibiotic use and obesity. In a large study of Danish children, antibiotics in the first 6 months of life were associated with increased risk of overweight at 7 years of age, but only among normal weight mothers [373]. Among overweight mothers, antibiotics slightly reduced risk for overweight in their children. However, antibiotic exposures were captured only for ear and lung infections, and the very low prevalence of antibiotic use under 6 months (7%) reported [373] compared to other studies in Denmark [387] and other high-income countries suggests antibiotic use may have been heavily under-reported.

In a study of UK children, exposure to antibiotics under 6 months of age was associated with increased BMI and risk of overweight and obesity at 3 years, but the effect did not persist at 7 years. Also, exposures between 6 and 23 months did not have a consistent effect on body mass [11]. In Philadelphia, antibiotic exposure in the first 2 years of life was associated with a minimal increase in overall risk for obesity from 2-5 years (Rate ratio: ~1.05), but the effect was larger in magnitude (Rate ratio: ~1.1) for greater number of antibiotic courses received, broad-spectrum antibiotics compared to narrow-spectrum and for earlier age (below 6 months) of first exposure compared to later age at exposure [228].

These human studies of the effects of antibiotic use on growth are complicated by the diverse indications for treatment across studies. In a small study of patients receiving long-term treatment with doxycycline for Q fever endocarditis, a quarter of treated patients showed abnormal weight gain [388]. Similar studies of patients with infective endocarditis also showed increases in BMI associated with long-term antibiotic treatment, but only among those receiving vancomycin [389,390]. It is unclear if these effects are specific to patients with endocarditis or if they are relevant to a general population.

Evidence that the microbiota affects growth is also supported by studies in which probiotic administration affects weight gain. Probiotics supplement the gut microbiota with organisms that are beneficial to the human host to create a healthier microbial community. The probiotics, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Lactobacillus ingluviei*, have been associated with weight gain in both animals and humans, while *Lactobacillus plantarum* and *Lactobacillus gasseri* have been associated with weight loss [391]. Several combinations of probiotics, sometimes in combination with milk formula or highly nutritious ready-to-use therapeutic food (RUTF), have also induced weight and/or height gain in children [360]. For example, a trial of infant formula supplemented with *Bifidobacterium breve* and *Lactobacillus rhamnosus* increased body weight and height in healthy infants [392]. On the other hand, different combinations of probiotics have also shown to reduce rates of weight gain and induce weight loss, suggesting the effects of probiotics on growth are complicated and likely organism-specific [360].

Because the functional repertoire of the microbiota includes energy harvest and fat deposition, different compositions of the microbiota may be more efficient in energy uptake than others and therefore contribute to weight change in humans [377–379]. However, while the

microbiota likely plays a role in growth, it is not clear which compositions or specific species in the microbiota are most beneficial, and it is difficult to predict the effects of alterations of the microbiota due to antibiotics or probiotics. The interaction of the microbiota with metabolism may be modulated by antibiotic use to cause either weight gain or weight loss [24]. The effects of antibiotic use on growth in association with treatment for common childhood illnesses are unknown.

Summary and rationale

Diarrhea is a universal and recurring disease during childhood that causes high morbidity and substantial mortality. The negative effects of diarrhea on malnutrition, growth, and cognition indicate the need for improved strategies for prevention and greater coverage of effective treatment. Inappropriate and ineffective antibiotic treatment for diarrhea and other childhood illnesses is widespread in India despite national and international recommendations against routine treatment with antibiotics. Antibiotics cause modifications in the gastrointestinal microbiota which may increase susceptibility to future infection and modify nutrient absorption and growth. However, longitudinal studies of childhood diarrhea have not considered the effects of antibiotics on diarrheal risk. While studies of diarrhea associated with antibiotic use have been completed, most focus on diarrhea occurring concurrently or soon after antibiotic use (AAD), and longer term effects of antibiotics on diarrheal risk have not been studied. In addition, these studies are often focused on hospitalized adults in high-income countries, and few studies have been completed among children in low-resource settings. Further, recent human studies of the effects of antibiotics on growth, and specifically obesity, have not consistently shown an effect or identified key components to explain this phenomenon. The effect of antibiotic treatment in

early childhood on growth has not been studied in a prospective observational study among children from LMICs.

To address these knowledge gaps, we aimed to assess the impact of antibiotic treatment among young children on diarrheal risk (Specific Aim 1) and growth outcomes (Specific Aim 2) before 3 years of age. We investigated the effects of antibiotic treatment of diarrhea, and any antibiotic exposures in the first 6 months of life, on future diarrhea, and estimated the impact of interventions that would prevent unnecessary antibiotic exposures. We also studied the effects of early life antibiotic exposures on both short and long-term growth. We hypothesized that the GI microbiota likely mediates the potential associations between antibiotics, diarrhea, and growth, as diagramed in Figure 2.1. Specifically, diarrhea or other illnesses result in antibiotic treatment, which modifies the microbiota and in turn affects immune system functioning. These changes may lead to increased susceptibility to subsequent diarrhea and result in poor growth. Microbiota modifications associated with antibiotic exposures may conversely also promote growth, given the established growth promoting effects of antibiotics in livestock and the association of antibiotics with obesity in humans.

Laboratory and epidemiologic studies support the hypothesized biological mechanism for the effect of antibiotics on diarrheal risk through modification of the microbiota. Antibiotic use causes dramatic reductions in diversity of the microbiota and alters the composition of bacterial species, especially during infancy when the developing microbiota is most susceptible to perturbations [24]. This corresponds to an important period in early childhood when a healthy microbiota is critical for gastrointestinal tract and immune system development. The microbiota of patients with diarrhea have altered compositions, suggesting that the microbiota plays a role in diarrhea and that diarrheal risk could be affected by perturbations through antibiotic exposure.

Similarly, the microbiotas differ between malnourished versus normal children, and lean versus obese adults. Small-scale studies have shown mixed results concerning the duration of the effect of antibiotics on the microbiota [18] such that some suggest the microbiota returns to a pre-treatment state within days or weeks of antibiotic exposure [286], while others show that antibiotics can cause long-lasting changes in the composition of the microbiota [17]. Therefore, it is plausible that antibiotic treatment could affect diarrheal risk and growth among children in both the short and long-term through modification of the microbiota, especially in a setting with high diarrhea incidence and overuse of antibiotics.

We focused on antibiotic treatment for diarrhea specifically (Aims 1A and 1C) and any antibiotic exposure regardless of clinical indication in the first 6 months of life (Aims 1B and 2). The first exposure is directly relevant to the effects of potential interventions concerning diarrhea treatment. Our observational (non-randomized) study is analogous to a hypothetical clinical trial in which children are randomized to treatment with antibiotics or not at each time a diarrhea episode arises. Since we are unable to recommend changes in all antibiotic prescription practices because many illnesses require treatment with antibiotics, focus on only unnecessary antibiotic exposures for diarrhea treatment in Aim 1C better corresponds to potential public health interventions.

On the other hand, because antibiotics for diarrhea may comprise a minority of total antibiotic exposures in children, an exploration of the effects of antibiotics regardless of clinical indication was also important to understand the basic etiology of the effects of antibiotics on diarrheal risk and growth. We focused on antibiotic use in the first 6 months since early life antibiotic exposure has the greatest impact on microbiota development and is likely to cause long-term changes in the microbiota [20,251,280,291,340]. Similarly, diarrhea during this time

causes longer-term growth deficits compared to diarrhea episodes at older ages, which suggests antibiotic exposures at this time may have the largest effect on growth outcomes. These two exposure definitions answer distinct yet complementary questions, one directly applicable to interventions and the other related to understanding general etiology.

Understanding the effects of antibiotic treatment for diarrhea will help inform policy makers, physicians, and public health professionals to improve treatment guidelines and rational antibiotic use. While rational use of antibiotics has been advocated to reduce the development of pathogen resistance to antibiotics, evidence of direct harm to children who are given antibiotics may accelerate the adoption of policies and practices to reduce inappropriate use. Such evidence would counter a commonly held assumption among doctors and caregivers that even if antibiotics are not strictly indicated, "at least they can't hurt." This impact could occur at multiple levels: 1) the results may provide an evidence base needed by policy makers to enforce regulations that control the sale of antibiotics; 2) physicians could incorporate this evidence into their cost-benefit equation when deciding whether to give children antibiotics, reducing prescription rates, and 3) mothers and caregivers, who rarely respond to appeals about the future development of pathogen resistance, would have an easily understood and logical reason to avoid giving antibiotics to their children. Reduction of inappropriate antibiotic use in these ways would benefit not only the individual children, but also society as a whole. The efficacy of antibiotics would be preserved for the treatment of more serious human infections, and the prevalence of drug-resistant bacteria may decrease [393,394]. This is of critical importance given the potential for the loss of the ability to treat more serious infections as bacteria become multidrug-resistant.

In addition, epidemiologic evidence that antibiotics increase risk for diarrhea will substantiate the rapidly accumulating laboratory-based evidence supporting a mechanism

through modifications of the microbiota. Further evidence of the effects of antibiotics on growth outcomes contributes to our understanding of the impact of inappropriate antibiotic treatment on long-term morbidity. Future follow-up studies may be developed from this study to assess the diversity and composition of the gut microbiota in stored stool samples from the children to better understand underlying biological mechanisms. These results may contribute in the future to the development of therapeutic and preventive interventions for diarrhea, such as those involving probiotics, that may stabilize and strengthen the microbiota.

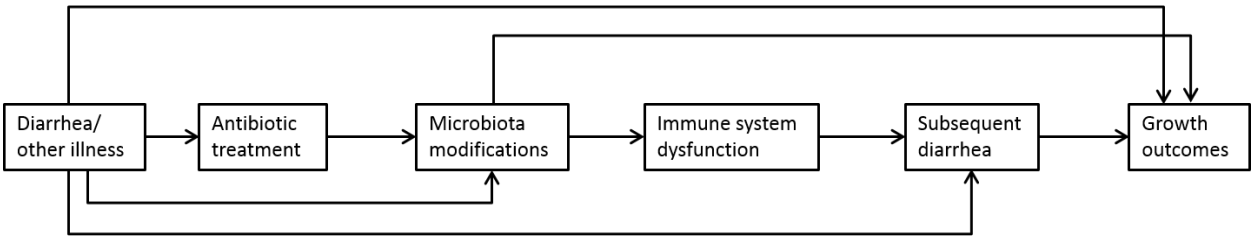

Figure 2.1. Conceptual diagram showing the hypothesized biological mechanisms for the causal pathways between antibiotic treatment, subsequent diarrhea, and growth. Potential confounders are omitted from this diagram for simplicity.

CHAPTER III: RESEARCH METHODS

Study design

We completed secondary analyses of existing data collected in three cohort studies of 452, 176, and 497 children respectively from semi-urban slums of Vellore, Tamil Nadu, India from 2002 to 2013 [28,395,396]. These cohorts provided highly detailed existing data on diarrhea incidence, duration, and severity, as well as record of antibiotics and other treatments for diarrhea. In two of the three studies, field workers visited enrolled children's homes twice-weekly from birth to 3 years of age and captured diarrhea incidence data based on a 3-day recall period. In the third study, children were followed weekly from birth to 2 years of age with 7-day recall. Study personnel recommended the children attend the study clinic when ill, and clinic records from these visits were linked to community follow-up data. Additional characteristics of each study are shown in Table 3.1.

All three studies were supervised under one principal investigator using the same protocols with minor adjustments. There was consistent quality control of data collection and management. Because all three cohort studies were completed in the same source population by the same investigators, data were comparable across studies. The three cohorts had a high incidence of diarrhea (approximately half had 4 or more episodes in the first 3 years of life), and approximately one-quarter of episodes were treated with antibiotics. By using existing data from a population with high incidence of diarrhea, the study was practical, feasible, and inexpensive to conduct.

Source population

The source population was all children born in geographically adjacent, semi-urban slums on the western side of the city of Vellore, in the state of Tamil Nadu, India (Figure 3.1). Tamil Nadu is a state with high immunization coverage and good public health care delivery infrastructure [397]. The slums of Vellore cover 2.2 km^2 and have approximate population densities of 17,000 per km^2. The rainy season occurs between August and November, and peak temperatures during the summer months reach above 40°C.

Residents form a relatively homogeneous population, and many families are long-term residents of the slums, which have less than 4% annual migration. Half of the households are Hindu, 45% are Muslim, and 5% are Christian. Manual production of tobacco-based cigarette-like products (*beedis*) for a daily wage is the most common occupation, while employment in unskilled work, domestic servitude, sweeping, and small trading is also common. Most households rely on the earnings from daily wages, without regular salaries or other benefits such as pensions and health insurance [395–397].

Rapid migration to urban areas in India in recent years has resulted in urban slum populations that are overcrowded, have poor housing conditions, and lack of clean water and sanitation infrastructure [396]. Tenancy and ownership of property is not secure, and houses are closely clustered with open drains and trash disposal [398]. Firewood is the primary cooking fuel in the slums of Vellore, and piped drinking water is supplied by the local municipality irregularly (at intervals of 2-28 days). This water is collected and stored in wide-mouthed containers and is often consumed without further treatment. Bore-wells and water tank trucks supplied by the Vellore Municipal Corporation provide alternative sources of drinking water when water is scarce [396]. Microbial contamination of the Vellore municipal water supply is common [399].

Residents have access to free government health services, including a physician-run urban health center (UHC) in the area and a government hospital ~5 km away. They can also access non-profit private health care providers, including the Christian Medical College Hospital (CMC) and its two outreach units—the Community Health and Development Hospital (CHAD) and the Low Cost Effective Care Unit (LCECU) [395]. Other private facilities, clinics, nursing homes, and traditional medicine and faith healers are also located in the vicinity. In addition, a physician-run clinic was established in the study area which provides free health care to study children. CMC study personnel have periodically completed health education campaigns in the study areas concerning the causes and outcomes of diarrhea in children and available treatment and prevention strategies. The infant mortality rate in this population estimated through community-based surveillance conducted by the UHC from 2008 to 2011 was 18.2 deaths per 1000 live births per year, and 38% of infant deaths were attributed to diarrhea from 1995-2003 [395,396].

This identified source population of young children was ideal for the proposed analyses since diarrhea incidence is highest in the first few years of life [2,40] and poor outcomes are associated with young age [30]. Regulation of antibiotics is low and correspondingly access to antibiotics is high in the slums of Vellore. Therefore, a substantial proportion of diarrhea episodes among children in this population experience were treated with antibiotics.

Study population

Information on study population, data collection, and laboratory analyses is summarized from published articles from the three cohorts and has been supplemented by discussions with the Principal Investigator and study team at CMC [28,62,171,395–410]. The study populations

consisted of children born in the study areas between March 2002 and August 2003, July 2008 and May 2009, and April 2009 and May 2010, for the three studies respectively. Women of child-bearing age were visited through repeated household surveys and identified at local antenatal clinics in the study areas to identify pregnancies (or pregnancies and children who were being exclusively breastfed for Study 2). Children of pregnant women were enrolled through consecutive recruitment following written informed consent obtained from each child's parent or guardian. Study 2 was a quasi-experimental study in which families received either bottled (n=90) or municipal (n=86) drinking water based on the street on which they lived. This cohort was not a birth cohort since children were recruited at birth or while they were still being exclusively breastfed. Inclusion and exclusion criteria across the three studies are compared in Table 3.2.

Collection of clinical and demographic data

Baseline information on demography (family size, number of siblings, sex, religion), socioeconomic indicators (socioeconomic status, maternal education, education and occupation of the head of the household), health-seeking behavior, environment, diet, and characteristics of delivery were collected within 45 days of birth. A score from the Kuppuswamy scale was assigned to each household as a measure of socioeconomic status (SES) based on educational and occupational level of the family, house ownership, total number of rooms in the house (excluding kitchen and bathroom), and household possessions. The scale ranges from 0-5; a score of 0 or 1 was considered low SES, 2 or 3 was considered middle SES, and 4 or 5 was considered high SES.

An assessment of water, food, and personal hygiene for each household was completed every six months through self-reported information and observation of: 1) treatment and storing of drinking water; 2) use of dedicated dippers to consume stored drinking water; 3) washing of foods, cooking vessels, and the breast prior to feeding; 4) hand washing before feeding the child and after defecation; 5) periodicity of bathing; and 6) details of toilets or other places of defecation. A household hygiene score ranging from 0-18, which has been previously validated in this population, was assigned based on inputs from the structured questionnaire. Households with scores at or above the upper tertile ($\geq$12) were considered to have good household hygiene. For our analyses, the hygiene measurement of each child recorded closest to their time of weaning was considered representative of hygiene across the follow-up period since variability in hygiene scores over time was low and hygiene at the time of weaning is most critical to diarrheal risk. Children who dropped out of the studies before weaning occurred were assigned the hygiene score recorded at baseline (Study 1 and 3) or closest to the time of drop-out (Study 2).

Birth weight and length were obtained from delivery records if available at the first home visit. Thereafter, heights and weights were measured each month of follow-up at the study clinic using single measurements. Weight was measured using a Salter weighing scale to the nearest 100 grams. Recumbent length was measured using a standard infantometer for the first year of life or until the child was able to stand, and subsequently height was measured with a stadiometer, both to the nearest millimeter. Relevant data types collected for each study are compared in Table 3.3.

At each twice-weekly visit (once-weekly for Study 2) to the households of enrolled children, field workers interviewed the caregiver about any illnesses on each day since the last

visit. If an episode of diarrhea was identified during the visit or through self-referral by the mother, the field worker visited the home daily to assess diarrhea severity details including the number of stools passed per day, consistency and color of stools, any associated fever or vomiting, treatment given, and diarrhea among other members of the family. Because accurate temperature measurements were not possible in the field, temperatures were recorded as normal, low-grade fever, and high-grade fever as reported by the caregivers. Details of hospitalization if applicable and medications given were also recorded, including the name of antibiotics given (recorded as free-response). The family was instructed to collect stool samples when diarrhea developed, and samples were collected every other day until three samples were collected or the episode ended (1-3 stool samples per diarrhea episode). A window period of 15 days (7 days before and 7 days after diarrhea) was allowed for collection of stool samples.

Regular home visits were also used as an opportunity to collect information on the incidence of other illnesses reported by the caregiver. Field workers encouraged the family to take the child to the study clinic for assessment of severity and appropriate treatment for diarrhea or for any illness caregivers felt might be serious. Field workers were also trained to identify other common morbidities by using standard definitions and to refer infants to a health facility if necessary. Children were referred to CHAD or CMC hospital when symptoms were severe, and illnesses were managed by physicians according to routine practice. The costs of care were covered by the study. Visits to other public and private healthcare facilities and physician-recorded diagnoses in prescription or discharge summaries were recorded if available at home visits.

Breastfeeding details were collected every two weeks until breastfeeding was stopped completely including the number of feeds per day and if liquid food, semi-solid food, and solid

food were given. In addition to samples collected during diarrhea, stool samples were collected from all children every 15 days (or monthly for Study 2). Exclusive breastfeeding was defined according to the standard WHO definition [411] as feeding with breast milk only with the exception of vitamins, mineral supplements, and medicines (no liquid, semisolid, or solid food).

Field workers were retrained and study protocols were standardized periodically over the study period. Anthropometric instruments were calibrated at least once a week. The data collected by field workers were validated in a 10% random subsample on revisits by the study supervisor and/or physician. Morbidity data at the study clinic were also used to validate the information gathered by the field workers. Missing data was monitored through completion of a missing data form by field workers at each time data were not collected. Dates and types of missing data, reasons for missing data, and information on whether the child had diarrhea at the time of missing data were recorded. Drop outs were accompanied by an assessment of reason for drop out and details of death if applicable.

Data were collected in standardized paper forms by field workers and double entered concurrently with data collection. Quality checks were completed at the time of data entry and through electronic logical checks before validation [397]. Because the proportion of missing data for baseline covariates was 5% or less, we imputed the median value for individuals with missing data. We assumed single imputation would result in negligibly over-precise confidence intervals given the proportion of missing values for these variables was small.

Case definitions

Assessment of diarrheal outcomes for Aim 1 was based on caregiver-reported diarrhea at home visits and by self-referral of caregivers to study personnel. We defined diarrhea as at least

three watery or loose stools in a 24-hour period. The episode ended on the day that the child's bowel movements returned to normal. Duration of a diarrhea episode was defined as the number of days from the first day of watery stools until the last day of watery stools inclusive. We defined a new episode of diarrhea as occurring only after at least 48 hours from the last episode during which bowel movements were normal. Person-time at risk was defined as all days during follow-up excluding days with diarrhea and the 48 hours after diarrhea during which a new episode of diarrhea could not be defined.

Severity of diarrhea was assessed at each day of illness using a modified version of the Vesikari scale, which was designed to assess severity of acute watery diarrhea caused by rotavirus in children. The scale was modified such that fever was reported by the mother instead of measured by a thermometer, and symptom inputs for the scale were assessed throughout the episode instead of solely at admission. This modified version has been used in this population previously for rotavirus-associated diarrhea and cryptosporidiosis [412]. The 20-point score is determined by the total duration of diarrhea, the maximum number of stools passed in 24 hours, the duration of vomiting (if present), the maximum number of vomiting episodes in 24 hours, fever (in °C), the degree of dehydration, and treatment. An episode was classified as mild if the score was between 1 and 5, moderate if the score was between 6 and 10, severe if the score was between 11 and 15, and very severe if the score was between 16 and 20.

The assessment of growth outcomes for Aim 2 was based on monthly anthropometrics taken at the study clinic. Steps to reduce bias due to measurement error were taken during data review completed at CMC. The standard deviation (SD) of the two measurements taken before and after each anthropometric measurement was calculated. Any measurements more than three SDs from these four measurements were recoded as missing. We also individually checked the

plausibility of measurements associated with the largest growth velocities between two measurements (top 1% of all such intervals). Implausibly large height or weight gains or losses in an interval resulted in the outlying measurement to be recoded as missing.

We used the 2006 WHO child growth standards as the reference population to calculate height-for-age (HAZ), weight-for-height (WHZ) and weight-for-age (WAZ) z-scores from the growth measurements. Children were classified as stunted (HAZ <−2 SD from the growth reference), wasted (WHZ <−2 SD), underweight (WAZ <−2 SD), or normal.

Exposure assessment

Assessment of antibiotic treatment for diarrhea as the exposure for Aim 1A was based on self-reported treatment information given by caregivers. Fieldworkers asked caregivers at the time of the current diarrhea episode to report all medications given during that specific episode. Questions were asked specifically about ORS, antimotility drugs, and antibiotics. The name of the drug(s) was recorded and a copy of prescription(s) was attached to the data collection form if available for reimbursement purposes. Field workers also asked about traditional medicines, including herbal, Homeopathic, and Unani medicines. Antibiotic exposures were classified by reviewing drug names reported and categorizing them by generic name and class of antibiotic. Because exposure information was reported at the time of the diarrhea episode (presumably during treatment), it is unlikely that the exposure was affected by recall bias. However, respondents may not have known the type or name of the specific drug given, resulting in misclassification.

While incidences of other illnesses among study children were recorded, treatments for these illnesses were not originally collected in the study protocols, and therefore antibiotic

exposures due to treatment for other illnesses were not available in the cohort datasets. For Aims 1B and 2, to assess the impact of any antibiotic exposures (regardless of clinical indication), we obtained antibiotic exposures for other illnesses from antibiotic prescriptions in study clinic records for Study 2 and 3. We reviewed clinic records for study children during the study period to record all treatments for diarrhea (including antibiotics and others) and all antibiotic prescriptions for non-diarrheal illnesses assessed at the study clinic. We extracted the date of clinic visit, diagnoses given, drug prescriptions including dosage and prescribed duration if available, and any other relevant treatment information. Complete record of antibiotic prescriptions was available only for Study 3 since one-third of prescriptions to children in Study 2 were not associated with a recorded Study ID number. We therefore restricted the analyses in Aims 1B, 1C, and 2 to Study 3. Exposure classification was derived from a combination of self-reported treatment information given by caregivers during diarrhea episodes and drug prescriptions for all illnesses from clinic records.

Sample size and participation rates

In Study 1, 914 pregnant women were identified and 452 children were sequentially found eligible and enrolled (Figure 3.2A). The most common inclusion criteria violation was that the mother did not intend to stay in the study area for 3 years, often because of a common cultural practice to relocate to the maternal village for several months after the birth of a child. Of 452 children enrolled, 391 children completed the first year of follow-up, 380 completed 2 years of follow-up, and 373 completed the study at 3 years. Five deaths occurred, including 3 that were associated with diarrhea and dehydration in the first year of life. The drop-out rate

across the three years was 17.5% and the median age at time of leaving the study was 4.4 months (interquartile range (IQR): 2.5, 33.6).

In Study 2, 193 pregnant women and exclusively breastfed children were identified and eligible for participation (Figure 3.2B). After the attrition and refusal of 17 subjects during the antenatal follow-up period, 176 children were enrolled. Because Study 2 allowed enrollment of children after birth while still exclusively breastfeeding, the median age at baseline was 22 days (IQR: 12.5, 56). Of 176 children enrolled, 170 children completed 1 year of follow-up and 160 completed two years of follow-up. The drop-out rate was 9.1% with a median age at dropout of 16.3 months (IQR: 7.7, 19.4). None of the study children died during the two year follow-up period.

In Study 3, 561 pregnant women were identified and eligible for participation (Figure 3.2C). Following attrition during the antenatal follow-up period due to refusal, migration, and adverse pregnancy outcomes, 497 children were enrolled in the study. Of these, 443, 420, and 410 children completed the first, second, and third study year respectively, resulting in a drop-out rate of 17.5% and median age at time of leaving the study at 7.8 months (IQR: 4.1, 15.5). Nine children died during follow-up; 3 deaths were associated with diarrhea. In all three studies, the most common reason for dropout was migration from the study area.

The total length of follow-up for the three cohorts was 1166.9, 311.6, and 1290.9 person-years respectively. The total numbers of diarrhea episodes reported during this follow-up time were 1955, 807, and 2295 episodes, of which 27.5%, 6.6%, and 23.5% were treated with antibiotics in the three studies respectively. Children in Study 1 had on average 28.9 weight measurements and 28.1 height measurements over the three years of follow-up. Children in Study 2 had on average 21.6 height and weight measurements over 2 years of follow-up, and

children in Study 3 had on average 30.6 height and weight measurements over 3 years of follow-up.

Ethical approval

The study was approved by the Institutional Review Boards of the Christian Medical College, Vellore, India, Tufts University Health Sciences campus, Boston, USA, and University of North Carolina – Chapel Hill, USA.

Table 3.1. Study design of three cohort studies of children in Vellore, Tamil Nadu, India 2002-2013.

	Study 1	Study 2	Study 3
Study period	2002-2006	2008-2011	2009-2013
Length of follow-up	3 years	2 years	3 years
Type of cohort	Birth	Quasi-experimental (Open cohort)	Birth
Frequency of follow-up	Twice weekly	Weekly	Twice weekly
No. of children enrolled	452	176	497

Table 3.2. Inclusion and exclusion criteria for three cohort studies of children in Vellore, Tamil Nadu, India 2002-2013.

	Study 1	Study 2	Study 3
Semi-urban slum areas (total population of study area)	Ramnaickanpalayam, Chinnallapuram, Kaspa (35,000)	Ramnaickanpalayam, Chinnallapuram, Kaspa, Vasanthapuram (40,000)	Ramnaickanpalayam, Chinnallapuram, Kaspa, Vasanthapuram (40,000)
Dates of identification of pregnant women*	November 2001-August 2002	September 2008-April 2009	March 2009-May 2010
Birth dates of enrolled children	March 2002-August 2003	July 2008-May 2009	April 2009-May 2010
End of follow-up	August 2006	May 2011	May 2013
Inclusion criteria	- Mother pregnant	- Mother pregnant or child exclusively breastfed	- Mother pregnant
Exclusion criteria	- Mother does not intend to remain in the area for 3 years - Birth weight <1500 g - Gross congenital anomalies - Residence in a brick-built house with five or more rooms	- Mother does not intend to remain in the area for 2 years - Birth weight <1500 g - Gross congenital anomalies	- Mother does not intend to remain in the area for 3 years - Birth weight <1500 g - Gross congenital anomalies - Serologically positive for HIV

*And recruitment of exclusively breastfed children in Study 2

Table 3.3. Clinical and demographic data collected from three cohorts of children in Vellore, Tamil Nadu, India 2002-2013.

Data Type	Study 1	Study 2	Study 3
Demographics and socioeconomic indicators	At baseline	At baseline	At baseline
Delivery details	At baseline	At baseline	At baseline
Anthropometrics	Monthly	Monthly	Monthly
Hygiene practices*	Monthly for first 6 months, then every 3 months	Every 3 months for first year, then every 6 months	Every 6 months
Breastfeeding practices	Every 2 weeks	Every 2 weeks	Every 2 weeks
Diarrhea incidence, duration, and severity; active surveillance for other illnesses	Twice weekly (3-day recall)	Weekly (7-day recall)	Twice weekly (3-day recall)
Antibiotic treatment for diarrhea	Yes	Yes	Yes
Type of antibiotic	No	No	Yes
Duration and dosage of antibiotic use	No	No	No

*Sanitation practices were collected less frequently in later cohorts due to low variability of responses over time

Figure 3.1. Map [413] indicating the geographic residence of the study population in Vellore (black point) in the state of Tamil Nadu (dark gray), India (white).

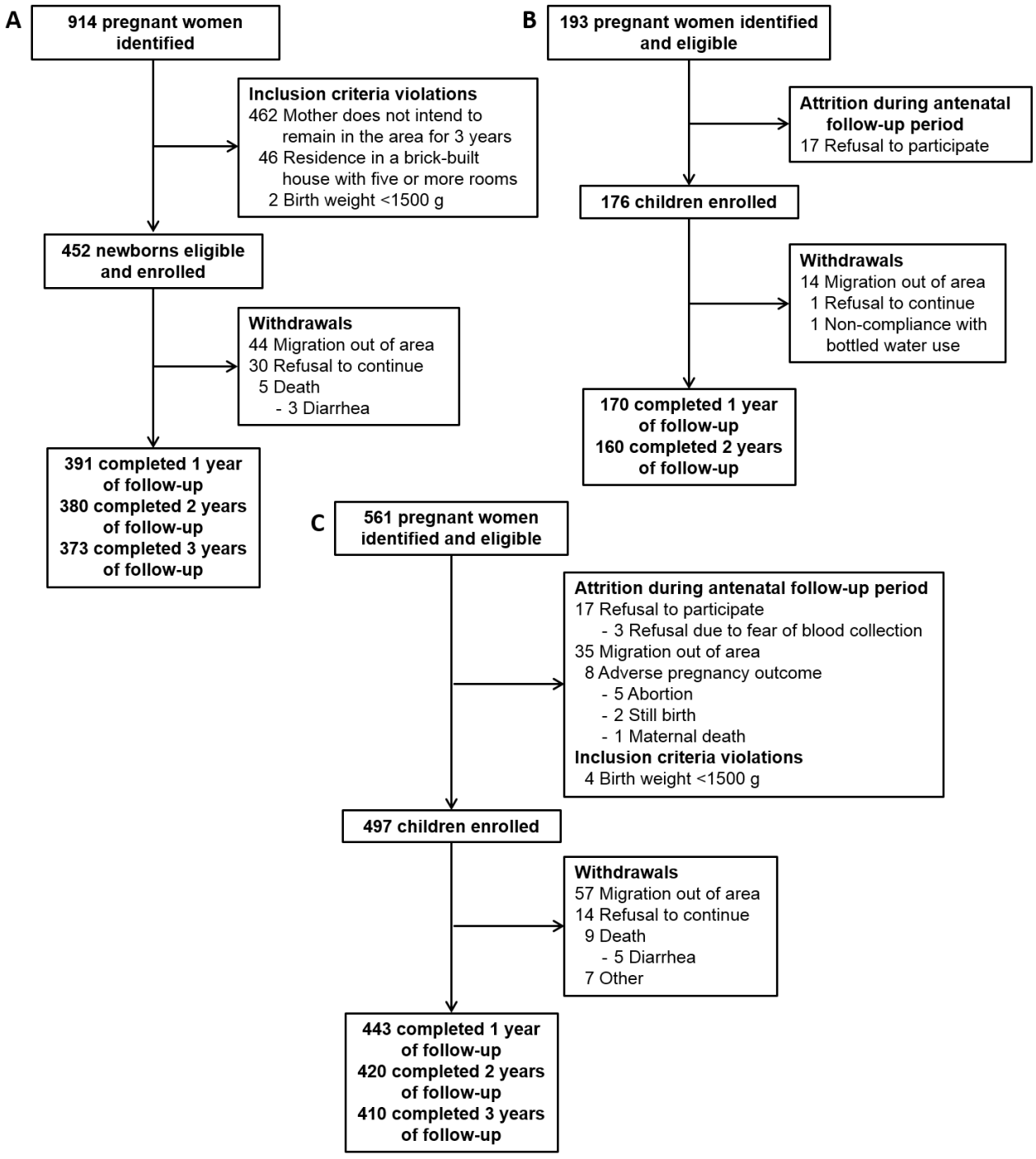

Figure 3.2. Summary enrollment and participation flowchart of the three study cohorts in Vellore, Tamil Nadu, India 2002-2013. **A –** Study 1; **B –** Study 2; **C –** Study 3. Sum of individual exclusions does not equal total exclusions where individuals were excluded for more than one reason.

CHAPTER IV: ANALYTIC METHODS

The statistical analysis methods varied for each aim to best answer the corresponding scientific questions of interest. In some cases, we applied relatively novel methods (Aim 1C) or adapted methods so that the results would be more interpretable with respect to our study questions (Aim 1A). In Aim 1A, we used inverse probability-weighted Kaplan-Meier (KM) curves to estimate differences in the time to subsequent diarrhea among children who received antibiotics for their most recent episode and those who did not. In Aim 1B, we used negative binomial regression to estimate the effect of any antibiotic exposure in the first 6 months of life on rates of diarrhea up to 3 years of age. In Aim 1C, we used the parametric g-formula with the same negative binomial model to estimate the impact of hypothetical interventions to reduce antibiotic use on diarrheal rates. To understand the effects of antibiotics on growth in Aim 2, we used longitudinal generalized linear regression with generalized estimating equations (GEE) and robust variance to account for within-subject correlation of the growth measurements. In short-term analyses, we also compared results from this model to those from the fixed-intercept model. We assessed both continuous (WAZ, HAZ, and WHZ z-scores) and binary (underweight, stunted, and wasted) growth outcomes with linear regression and the Poisson approximation to log-binomial regression respectively. The cohort data from Study 3 was used in the primary analyses for Aim 1A, and exclusively for Aims 1B, 1C, and 2, since this was the only cohort with complete information on antibiotic treatment for non-diarrheal illnesses.

Aim 1A

Analyses of the effect of antibiotic treatment for diarrhea on risk of subsequent diarrhea episodes was restricted to children who experienced at least one episode of diarrhea and therefore had the opportunity to be exposed. We included 434 of 497 (87.3%) children in Study 3, 160 of 176 (90.9%) in Study 2, and 390 of 452 (86.3%) in Study 1 who had at least one diarrhea episode. We focused the analyses on Study 3, which was the most recent cohort and had complete information on antibiotic treatment for non-diarrheal illnesses. Because Study 1 and 2 lacked complete records of antibiotics, and the type of antibiotics given for diarrhea were unknown, we presented the results from these cohorts as sensitivity analyses.

The primary exposure was antibiotic treatment for diarrhea based on caregiver-report during the episode. To validate caregiver-report, we also used alternative definitions of exposure to antibiotics. First, we restricted the exposed group to only those children whose caregivers reported the name of a confirmed antibiotic in the free-response section of the questionnaire. Second, we considered children exposed if either their caregiver reported antibiotics were given (by indicating yes/no) or if an antibiotic prescription was recorded in clinic records during the diarrhea episode. Finally, we considered children exposed only if a confirmed antibiotic name was reported or if a prescription was recorded in the clinic records.

We used logistic regression to calculate inverse probability of exposure weights stabilized by the marginal probability of exposure [414]. Confounding variables for the exposure model were chosen by causal directed acyclic graph (DAG; Figure 4.1) [415] to account for baseline characteristics and indications for treatment. Continuous variables were modeled flexibly with restricted quadratic splines [416], and covariate specifications were compared by Akaike's information criterion. Final covariates selected and their specifications are shown in

Table 4.1. To remove extreme weight values [417], weights were censored at the 0.5^{th} and 99.5^{th} percentiles by resetting the value of weights greater than the 99.5^{th} percentile and less than the 0.5^{th} percentile to the values of the 99.5^{th} and 0.5^{th} percentiles respectively.

We estimated inverse probability-weighted KM curves [414,418] for the time to next diarrhea episode comparing children who did and did not receive antibiotics for the previous episode. The time scale [418] was from 48 hours after the previous diarrhea episode to the incident day of the next episode. Children were censored at drop-out, death, or the end of follow-up at 3 years of age. We assumed person-time during which children were temporarily unreachable was missing at random and drop-out was non-informative given the small proportion of drop-outs (n=50, 11.5% overall; n=18, 4.1% between the first and second diarrhea episode). We calculated the time difference and time ratio at 50% diarrhea-free survival, the median survival time, from the weighted KM curves. Confidence intervals were constructed by bootstrap [419] with 200 resamples at the level of the individual to account for clustering of episodes within children.

We also estimated hazard ratios comparing the same exposure groups using marginal structural Cox models [418] with the same inverse probability weights. These models were estimated by pooled logistic regression with adjustment for time using a restricted quadratic spline [416]. Correlation between outcomes from the same child was accounted for using generalized estimating equations with a robust variance estimator.

We assessed modification of the effect of antibiotics by age at exposure by stratification. We also considered the effect of specific antibiotics commonly given (cotrimoxazole and cefixime) by comparing children receiving each drug with children given no antibiotics. To assess the impact of long episode duration contributing to shorter time between episodes, we

repeated the main analyses excluding all episode pairs where the first episode lasted for more than 7 days.

To assess whether antibiotics were associated with the severity of subsequent diarrhea when another episode occurred, we estimated the effects of antibiotic treatment for the previous episode on the severity and duration of the next episode. In models weighted for the same covariates as in above analyses, we used inverse probability-weighted linear regression with the Vesikari score and number of days with diarrhea as continuous outcomes. We also estimated the adjusted relative risk for a severe (Vesikari $\geq$ 11) and prolonged/persistent ($\geq$7 days) next episode using inverse probability-weighted log-binomial regression.

Aim 1B

To assess whether any antibiotic exposure in the first 6 months of life affected subsequent rates of diarrhea from 6 months to 3 years of age, we restricted analyses to Study 3, which had complete information on antibiotics given for non-diarrheal illnesses. We included 465 of 497 (93.6%) children in Study 3 who remained in the study for more than 6 months and were therefore at risk for diarrhea after 6 months of age. We did not restrict to children with at least one diarrhea episode since diarrhea was not a prerequisite of antibiotic exposure in this analysis.

The main exposures were any antibiotic exposure in the first 6 months of life, as well as the total number of antibiotic courses in the first 6 months of life, both based on antibiotic prescriptions recorded in clinic records and caregiver-reported antibiotic treatment at birth and for diarrhea. We excluded all topical antibiotics (neosporin, neomycin, soframycin, and gentian violet). Rates of diarrhea after 6 months of age per child were defined by the total number of incident episodes divided by the total time that child remained in the study. We excluded from

person-time denominators days with diarrhea (when a child was not at risk of incident diarrhea), periods during which the child was unreachable, and any time after loss to follow-up or death.

We used Poisson and negative binomial regression to model the rates of diarrhea from 6 months to 3 years of age. Crude and adjusted incidence rate ratios (IRRs) for diarrhea were estimated comparing children who were exposed to early life antibiotics to those who were not. Confounding variables were chosen using the DAG [415] (Figure 4.1), and optimal variable coding was determined by likelihood ratio test (α=0.1) and Akaike's information criterion. Final covariates selected and their specifications are shown in Table 4.2. We assessed effect measure modification by exclusive breastfeeding, sex, Cesarean section birth, age at first diarrhea, and growth status (underweight, stunted, wasted) in first 6 months by reporting stratum-specific estimates and testing homogeneity by likelihood ratio test (α=0.1). We further explored the role of breastfeeding by assessing the crude association between exclusive breastfeeding and antibiotic treatment using log-risk and linear regression.

To assess potential misclassification of the exposure, we repeated main analyses with more restricted definitions of antibiotic exposure that included caregiver-reported antibiotics only if an antibiotic name was recorded. To determine if the effect of antibiotics on diarrheal rates differed by antibiotic type, we repeated analyses separately comparing children who exclusively received one of the most commonly used antibiotics, amoxicillin and cotrimoxazole, to children who received no antibiotics. We further assessed if the effect of antibiotics differed depending on 1) the indication for which antibiotic treatment was given; 2) the number of diarrhea episodes experienced in the first 6 months of life; and 3) the time period for diarrheal outcomes (6-18 months of age compared to 18-36 months).

Aim 1C

To estimate the effect of hypothetical interventions to reduce unnecessary antibiotic use, we used the same data and model structure as in Aim 1B (Table 4.2). We classified potentially unnecessary antibiotic use by characterizing antibiotic treatments under 6 months of age by indicating diagnosis: non-bloody diarrhea, URI, non-diarrheal acute gastroenteritis (AGE; i.e. vomiting), and other, which included bloody diarrhea. We considered antibiotics for non-bloody diarrhea as "not indicated" according to clinical guidelines given our confidence in the diarrhea case definition. We considered antibiotics for URI and non-diarrheal AGE as "likely not indicated" to reflect the potential variability and uncertainty in diagnoses from the study clinic records. Antibiotics given for all other illnesses, including cases of bloody diarrhea, were considered necessary. We considered two interventions: (i) removing all antibiotics that were not indicated, and (ii) removing all antibiotics that were not indicated or likely not indicated. All other antibiotic exposures were not affected by the interventions. Given our binary exposure classification (exposed to at least one course of antibiotics versus none), children remained exposed to antibiotics if they had any necessary antibiotic exposures. Children who received only unnecessary antibiotics moved from exposed to unexposed after the interventions. The targeted interventions were applied only to children who had already stopped exclusive breastfeeding.

We used the parametric g-formula [420–425] to estimate contrasts associated with the effect of antibiotic use on diarrheal rates. The general procedure was as follows:

1. Estimate beta coefficients for the observed exposure and covariates using the negative binomial model with rates of diarrhea from 6 months to 3 years as the outcome
2. Use the estimated coefficients to predict the outcome in all individuals under the index exposure and again under the referent exposure

3. Average the predicted outcomes across individuals in the exposure groups
4. Compare the average outcomes to estimate the adjusted rate difference
5. Estimate the number needed to treat (NNT) as the reciprocal of the rate difference
6. Construct confidence intervals by bootstrap with 200 replicates [419]

Using this method, we estimated the population average causal effect, population attributable contrast, generalized impact contrast, and the targeted impact contrast. In sensitivity analyses, we also estimated the population average and generalized impact contrasts in the exposed population only (commonly termed the "effect of treatment in the treated"). We also expanded our models to estimate separate coefficients for the effects of necessary and unnecessary antibiotics and included the interaction between them to account for any differences in effect by indicating condition.

Aim 2

To assess whether antibiotic exposure affects growth in the first 3 years of life, we restricted analyses to Study 3, which had complete information on antibiotics given for non-diarrheal illnesses. We included all 497 children in the parent cohort for short-term analyses of effects in the first 6 months of life. In the long-term analyses, we included 456 (91.8%) children who remained in the study until at least 6 months of age and had one or more growth measurements after 6 months of age. Growth z-scores were considered the primary outcomes of interest since they vary linearly with age and account for growth differences by age and sex. These models were simpler to fit compared to modeling absolute height and weight, which requires the inclusion of higher order terms for age to capture the non-linearity of growth curves. To improve the interpretability of effects, we also translated the effects on z-scores to their age

and gender-specific equivalents in height and weight using the one standard deviation differences in weight/height from the expanded z-score tables provided by the WHO [426,427].

Short-term effects

We considered growth measurements taken within one week before or after a child's monthly birth anniversary as their weight/height at that month of age. Growth measurements for months during which a child was not measured during this two week period were considered to be missing for that child (6.5% of child-months overall).

We used longitudinal general linear regression to model WAZ, HAZ, and WHZ in monthly intervals from 0 through 5 months of age. We estimated the effects of antibiotic exposures in a given month on WAZ, HAZ, and WHZ at the end of the following month (conceptually depicted in Figure 4.2A), and accounted for correlation between outcomes from the same child using GEE with a robust variance estimator. To assess the sensitivity of results to the time period between antibiotic exposure and outcome, we repeated the monthly analyses with outcomes both at the end of the exposure month (Figure 4.2B) and at two months following the exposure month (Figure 4.2C).

Confounding variables for the exposure model were chosen using the DAG [415] (Figure 4.1) to account for baseline growth status, other baseline characteristics, and indications for treatment, which are the most important determinants of antibiotic use and also affect child growth. Optimal variable coding was determined by the quasi-likelihood under the independence model criterion (QIC), which is appropriate for GEE models [428]. Final covariates selected and their specifications are shown in Table 4.3. We stratified effects by month of antibiotic exposure,

gender, exclusive breastfeeding in the exposure month, baseline malnutrition status of the child (underweight, stunted, or wasted), and illness burden.

To validate our results with an alternate model that eliminates potential unmeasured child-level confounding, we used a fixed-intercept model in which the effects of antibiotic use in monthly intervals were estimated within-child (a child's exposed and unexposed months served as the index and reference exposures respectively), and between-child heterogeneity was captured in fixed child-specific effects (not estimated in the model) [429]. We again estimated the effect of antibiotic exposure in a given month on WAZ, HAZ, and WHZ at the end of the following month and stratified effects by gender. We used the robust variance estimator to account for correlation between observations within-child and necessarily included only the time-varying covariates [429] listed above.

Long-term effects

We used longitudinal general linear regression with GEE to model all WAZ, HAZ, and WHZ measurements after 6 months of age as a function of antibiotic use in the first 6 months of life. Each child's exact age in days at the growth measurement was retained in the longitudinal models. We included the growth z-score corresponding to the outcome at 6 months as a covariate to ensure the estimation of long-term effects of antibiotics on growth rates following 6 months of age. Baseline confounding variables were again chosen by using the DAG [415] (Figure 4.1) and were largely the same as those in the short-term analysis, but also included Cesarean section birth. Final covariates selected and their specifications are shown in Table 4.3. We stratified effects by sex, number of antibiotic courses received, and age period of growth (6 months-1 year,

1-2 years, 2-3 years). We further assessed modification of effects by exclusive breastfeeding, illness burden, and malnutrition status.

For both short and long-term analyses, we estimated the effects of antibiotics on the relative risk of underweight, stunting, and wasting with the same exposure groups and covariates as the linear regression models. We used Poisson regression with the robust variance estimator as an approximation of log-binomial regression [430] since the log-binomial regression models did not converge. We also validated results by repeating analyses with the same alternative definition of antibiotic exposure used in Aim 1 analyses.

Table 4.1. Covariate specification in final adjusted models for Aim 1A.

Covariate	**Model specification**
Diarrhea episode number	Indicator variables for episode 2, 3, 4, and 5+
Child sex	Dichotomous
Socioeconomic status	Indicator variables for low and medium/high based on the Kuppuswamy scale [431]
Maternal education	Linear continuous
Cesarean birth	Dichotomous
Low birth weight (<2.5 kg)	Dichotomous
Preterm birth (<37 weeks)	Dichotomous
Hospitalization at birth	Dichotomous
Antibiotics given at birth	Dichotomous
Age at previous episode	Restricted quadratic spline [416] with knots at the 20^{th}, 40^{th}, 60^{th}, and 80^{th} percentiles
Vesikari score[412] of the previous episode	Restricted quadratic spline with knots at the 25^{th}, 50^{th}, 75^{th} percentiles
Duration of previous episode	Restricted quadratic spline with knots at the 5^{th}, 50^{th}, 95^{th} percentiles
Hospitalization during previous episode	Dichotomous
Fever during previous episode	Linear continuous
Dehydration during previous episode	Dichotomous
Bloody diarrhea during previous episode	Dichotomous
Zinc given during previous episode	Dichotomous
Underweight at previous episode (weight-for-age z-score <−2 SD)	Dichotomous
Stunted at previous episode (height-for-age z-score <−2 SD)	Dichotomous
Wasted at previous episode (weight-for-height z-score <−2 SD)	Dichotomous
Exclusive breastfeeding at previous episode	Dichotomous
Any breastfeeding at previous episode	Dichotomous
Number of previous antibiotic courses for any illnesses	Linear continuous
Number of sick days between episodes	Restricted quadratic spline with knots at the 25^{th}, 50^{th}, and 75^{th} percentiles
Other antibiotics given between episodes	Dichotomous

Table 4.2. Covariate specification in final adjusted models for Aims 1B and 1C.

Covariate	**Specification**
Child sex	Dichotomous
Socioeconomic status	Indicator variables for low, medium, and high based on the Kuppuswamy scale [431]
Maternal education	Linear continuous
Household hygiene	Restricted quadratic spline [416] for continuous hygiene score [432] with knots at the 25^{th}, 50^{th}, 75^{th} percentiles
Household crowding (number of household members/number of rooms)	Linear continuous
Low birth weight (<2.5 kg)	Dichotomous
Exclusive breastfeeding at 6 months of age	Dichotomous
Number of diarrhea episodes in first 6 months	Disjoint indicators for 1, 2, 3, 4, and 5+ episodes
Total number of days with diarrhea in first 6 months	Restricted quadratic spline [416] with knots at the 25^{th}, 50^{th}, 75^{th} percentiles
Maximum Vesikari score[412] of any diarrhea episode in first 6 months	Linear continuous
Number of severe episodes in first 6 months (Vesikari $\geq$ 11)	Linear continuous
Prolonged or persistent diarrhea episode in first 6 months	Dichotomous
Hospitalization for diarrhea in the first 6 months	Dichotomous
Fever during diarrhea in first 6 months	Dichotomous
Dehydration during diarrhea in first 6 months	Disjoint indicators for 0, 1, and 2+ diarrhea episodes with dehydration
Underweight (average weight-for-age z-score under 6 months of age <−2 SD)	Dichotomous
Stunted (average height-for-age z-score under 6 months of age <−2 SD)	Dichotomous
Wasted (average weight-for-height z-score under 6 months of age <−2 SD)	Dichotomous
Any severe illness in first 6 months	Dichotomous
Number of other infections in first 6 months	Linear continuous

Table 4.3. Covariate specification in final adjusted models for Aim 2.

Short-term models	
Covariate	**Specification**
Month of age corresponding to exposure period	Indicator variables for months 0, 1, 2, 3, 4, and 5
Baseline z-score (at beginning of exposure month)	Continuous
Child sex	Dichotomous
Socioeconomic status	Indicator variables for low and medium/high based on the Kuppuswamy scale [431]
Maternal education	Dichotomous: 0-12 years (no formal education, primary/middle) vs. 13+ years (college/polytechnic/professional)
Household hygiene	Linear continuous hygiene score [432]
Household crowding (number of household members/number of rooms)	Dichotomous: 0-<5 people/room vs. ≥5 people/room
Low birth weight (<2.5 kg)	Dichotomous†
Preterm birth (<37 weeks)	Dichotomous§
Cesarean birth	Dichotomous*§
Total number of days with diarrhea in exposure month	Indicator variables for 0 days, 1-3 days, and >3 days*
Severe diarrhea in exposure month (Vesikari ≥ 11)	Dichotomous§
Hospitalization in exposure month	Dichotomous
Dehydration during diarrhea in exposure month	Dichotomous
Prolonged or persistent diarrhea episode in exposure month	Dichotomous
ORS received during diarrhea in exposure month	Dichotomous
Exclusive breastfeeding in exposure month	Indicator variables for none, part of the month, full month*†
Total days severely ill or with other infections in exposure month	Indicator variables for 0 days, 1-7 days, and >7 days
Total number of days with of diarrhea in previous month	Indicator variables for 0 days, 1-3 days, and >3 days
Including interaction term with month of age for: *WAZ model, †HAZ model, §WHZ model	
Fixed-intercept model	
Baseline z-score	Continuous
Total number of days with in exposure month	Indicator variables for 0 days, 1-3 days, and >3 days
Severe diarrhea in exposure month (Vesikari ≥ 11)	Dichotomous
Hospitalization in exposure month	Dichotomous

Dehydration during diarrhea in exposure month	Dichotomous
Prolonged or persistent diarrhea episode in exposure month	Dichotomous
ORS received during diarrhea in exposure month	Dichotomous
Exclusive breastfeeding in exposure month	Indicator variables for none, part of the month, full month
Total days severely ill or with other infections in exposure month	Indicator variables for 0 days, 1-7 days, and >7 days
Duration of diarrhea in previous month	Indicator variables for 0 days, 1-3 days, and >3 days
Long-term models	
Baseline z-score (at 6 months of age)	Continuous
Child sex	Dichotomous
Socioeconomic status	Indicator variables for low and medium/high based on the Kuppuswamy scale [431]
Maternal education	WAZ/WHZ models: Dichotomous: 0-12 years (no formal education, primary/middle) vs. 13+ years (college/polytechnic/professional) HAZ model: Indicator variables for 0 years (no formal education), 1-8 years (primary/missle), and 9+ years (higher secondary/college/ polytechnic/professional)
Household hygiene	Dichotomous: Poor (hygiene score <12) vs. good (hygiene score ≥12 [432])
Household crowding (number of household members/number of rooms)	WAZ/HAZ models: Dichotomous: ≤2 people/room vs. >2 people/room WHZ model: Indicator variables for ≤2 people/room, 2.1-4.9 people/room, and ≥5 people/room
Low birth weight (<2.5 kg)	Dichotomous
Preterm birth (<37 weeks)	Dichotomous
Cesarean birth	Dichotomous
Exclusive breastfeeding until at least 3 months of age	Dichotomous
Total number of days with diarrhea in first 6 months	Indicator variables for 0 days, 1-14 days, and >14 days
Prolonged or persistent diarrhea episode in first 6 months	Dichotomous
Maximum Vesikari score [412] of any diarrhea episode in first 6 months	Quadratic
Fever during diarrhea in first 6 months	Dichotomous
Dehydration during diarrhea in first 6 months	WAZ/WHZ models: Dichotomous HAZ model: Indicator variables for 0, 1, and 2+ diarrhea episodes with dehydration

Days with other infections in first 6 months	Indicator variables for 0 days, 1-14 days, and >14 days
Any severe illness in first 6 months	Dichotomous
Underweight (average weight-for-age z-score under 6 months of age <−2 SD)	Dichotomous (HAZ model only)
Stunted (average height-for-age z-score under 6 months of age <−2 SD)	Dichotomous (WAZ model only)

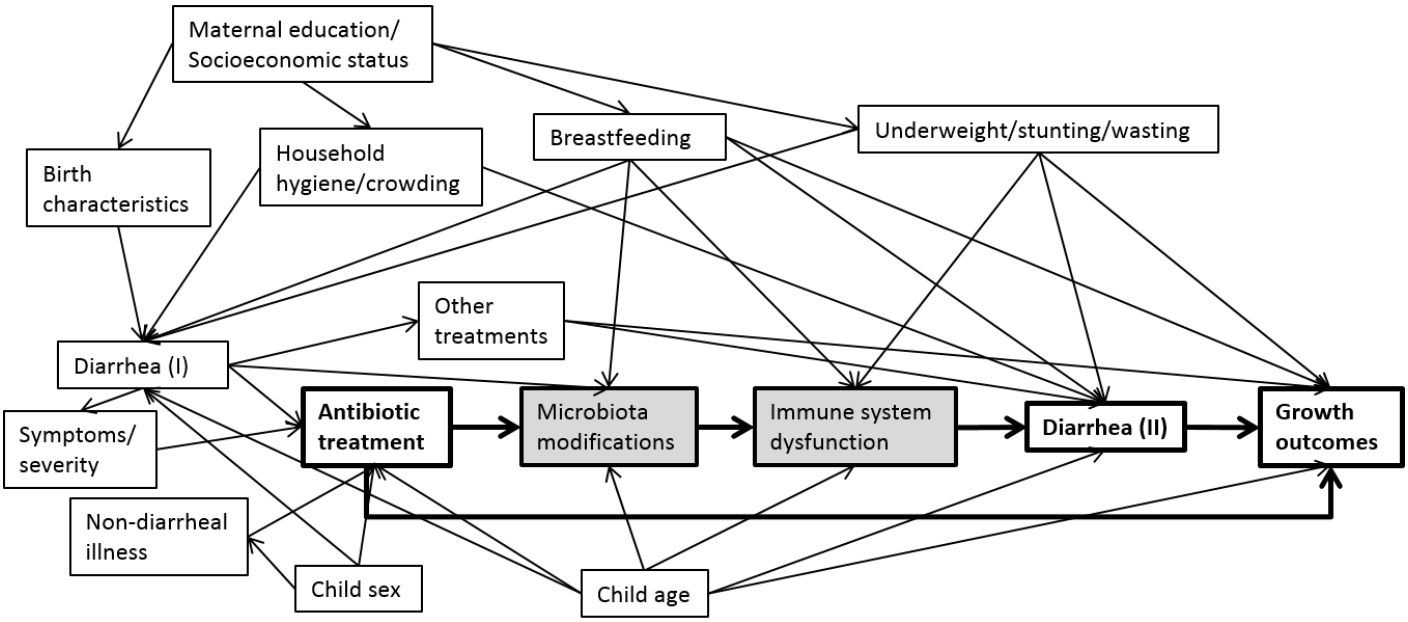

Figure 4.1. Directed acyclic graph (DAG) for the effects of antibiotics on diarrhea and growth. Bold indicates main exposure or outcome; heavy black lines indicate causal paths of interest; variables shaded in grey are unmeasured. Birth characteristics include cesarean birth, pre-term birth, low birth weight, hospitalization, and antibiotics at birth. Other treatments include zinc and oral rehydration salts (ORS).

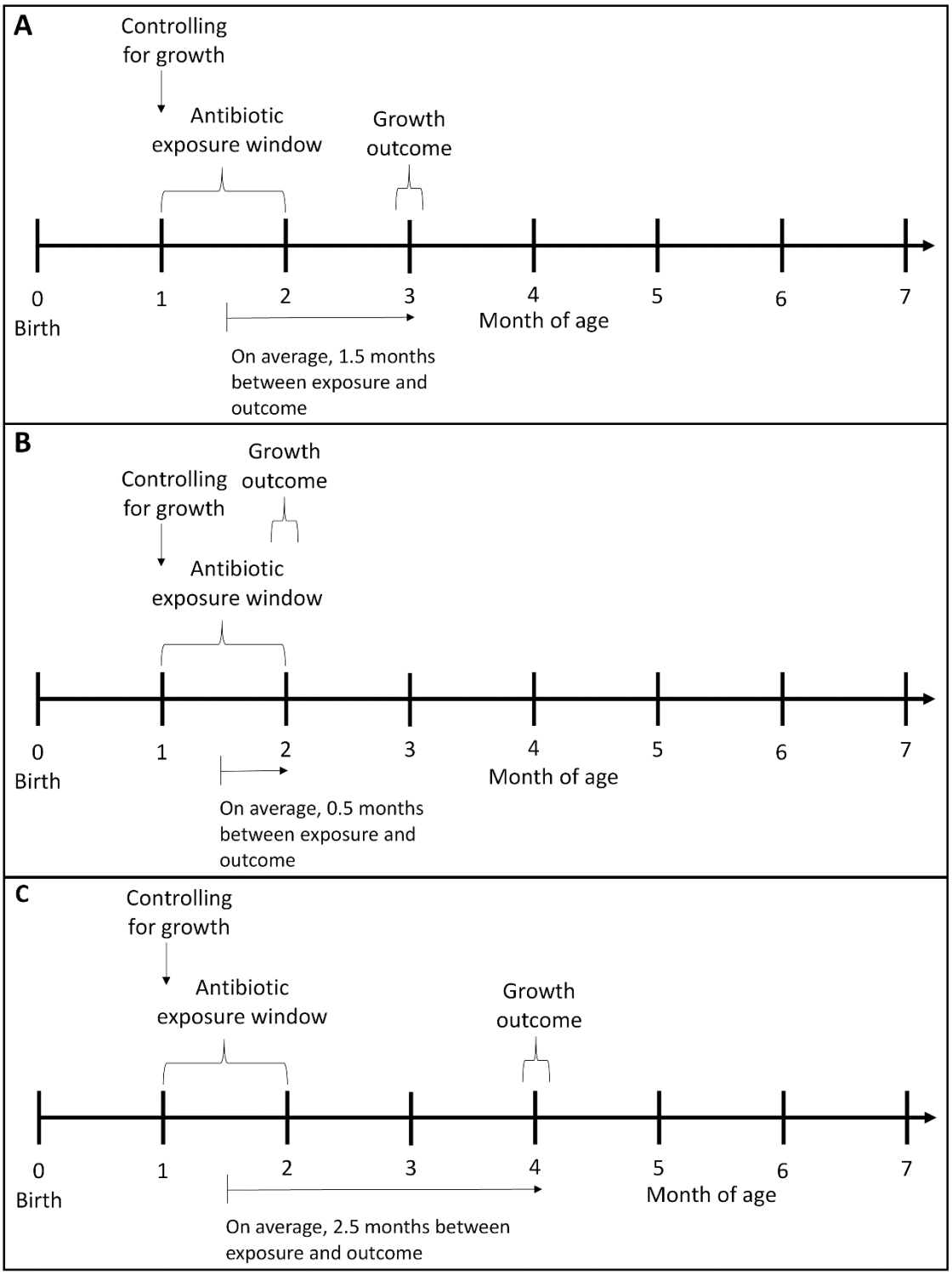

Figure 4.2. Schematic of exposure period and age of outcome assessment for short term growth analyses. Also indicated are the age of baseline growth measurement included in the models and the average time between exposure and growth outcome. The analyses included the analogous scheme for all months through 6 months of age. **A** – Primary analysis; **B** & **C** – Sensitivity analyses.

CHAPTER V: ANTIBIOTIC TREATMENT OF DIARRHEA IS ASSOCIATED WITH DECREASED TIME TO THE NEXT DIARRHEA EPISODE AMONG YOUNG CHILDREN IN VELLORE, INDIA

Abstract

Background

Antibiotics are commonly given for the treatment of childhood diarrhea, but are not indicated in most cases. Antibiotics modify the gastrointestinal microbiota, which may have unanticipated effects on the risk of subsequent diarrhea.

Methods

In a prospective observational cohort study, we assessed the effect of caregiver-reported antibiotic treatment for diarrhea on the timing of a child's next episode among 434 children followed from birth to 3 years of age in Vellore, India. We estimated median time differences and time ratios from inverse probability of exposure-weighted Kaplan-Meier curves for the time to next diarrhea episode comparing children who did and did not receive antibiotics for the previous episode.

Results

Study children had more than 5 diarrhea episodes on average in the first 3 years of life, and more than a quarter of all episodes were treated with antibiotics. Children who received antibiotics for their first diarrhea episode had their second episode on average 8 weeks earlier (median time difference: -8, 95% CI: -10, -3) than children who did not receive antibiotics. The

effects of antibiotics on subsequent diarrhea were greatest at earlier episodes and younger ages, and cefixime had a slightly larger effect compared to cotrimoxazole.

Conclusions

Antibiotic treatment of diarrhea was associated with reduced time to a subsequent diarrhea episode, especially among younger infants. While rational use of antibiotics has been advocated to reduce antimicrobial resistance in a population, we show that overuse of antibiotics may also have a direct adverse effect on individual patients.

Introduction

Diarrhea is a universal and recurring disease during childhood with the highest burden in low and middle-income countries. In 2010, the global incidence of diarrhea before age 5 was estimated to be 2.7 episodes per child-year, which corresponds to approximately 1.7 billion total episodes and resulted in 700,000 deaths [30].

Antibiotics are widely accessible and commonly used for the treatment of childhood diarrhea in India. However, international and Indian organizations, including the World Health Organization, recommend against routine use of antibiotics to treat diarrhea [10,188]. Antibiotics are generally contraindicated for non-bloody diarrheas because they are ineffective against non-bacterial and resistant pathogens, and most episodes of diarrhea are self-limiting [189,191]. Despite these arguments, several healthcare facility-based studies in India have reported antibiotic prescription rates for acute childhood diarrhea from 50-90% [9,199,203,204]. In a nationwide community-based survey, 16% of children under 5 who had diarrhea in the two weeks preceding survey reported treatment with antibiotics, and another 30% reported treatment with unknown drugs that may have included antibiotics [8].

While major concerns about inappropriate antibiotic use often focus on the development of pathogen resistance to antibiotics, direct harm to patients is also possible and often overlooked [15]. Specifically, antibiotics may disrupt the GI microbiota—the complex community of microorganisms inhabiting the human GI tract—by causing a sharp reduction in the abundance and diversity of organisms [14,20]. This disruption can be prolonged, and the recovery of the microbiota following antibiotic exposure is often incomplete [16,17]. The microbiota is important for the development of the immune system [265,279], and may protect against diarrheal disease by occupying intestinal mucosal sites and inhibiting the attachment and growth of pathogens [296,433,434].

Studies of the impact of antibiotics on diarrhea most often focus on the incidence of AAD occurring within 8 weeks of antibiotic exposure [229,230], and often among hospitalized adults in high-income countries [235]. Longitudinal investigation of the effects of antibiotics on diarrheal risk has not been completed among children in resource-poor settings. In a birth cohort of children from Vellore, India, we assessed the effect of antibiotic treatment for diarrhea on the timing of a child's next diarrhea episode.

Methods

We analyzed data from a prospective observational cohort study on immune responses to cryptosporidiosis in 497 children followed from birth to 3 years of age from 2009 to 2013. The study population, enrollment strategy, and data collection methods have been described previously [28]. Briefly, baseline information on demography, socioeconomic indicators, health-seeking behavior, environment, diet, and characteristics of delivery were collected within 45 days of birth. Fieldworkers interviewed caregivers twice per week about any illnesses since the last visit. Clinical characteristics of the diarrhea episodes were recorded including the number of

stools per day, consistency and color of stools, fever or vomiting, associated hospitalization, and treatments given. Heights and weights were measured monthly at the study clinic, and breastfeeding histories (exclusive, non-exclusive, none) were collected every two weeks until breastfeeding was stopped completely.

Data and definitions

Diarrhea was defined using the standard WHO definition as at least three loose or watery stools in a 24-hour period [10]. Duration of a diarrhea episode was defined as the number of days from the first day of watery stools until the last day of watery stools inclusive. A new episode of diarrhea was defined only after at least 48 hours of normal bowel movements since the previous episode. Person-time at risk was defined as all days during follow-up excluding days with diarrhea and 48 hours after an episode of diarrhea during which a new episode of diarrhea could not be defined.

Severity of diarrhea was assessed using the 20-point Vesikari scale [412]. Episodes were classified as mild (1-5), moderate (6-10), severe (11-15), and very severe (16-20). Episodes were classified as acute if lasting 0-6 days or prolonged/persistent if lasting for 7 or more days.

The primary exposure was antibiotic treatment for diarrhea based on caregiver-report during the episode. A yes-no question was asked specifically about whether antibiotics were given and the name of the drug(s) was recorded if known (available for 64.0% of antibiotic reports). We also extracted antibiotic prescriptions from clinic records for all illnesses (most commonly respiratory, skin, and ear infections) assessed at the study clinic.

Children were classified according to standard definitions as underweight (weight-for-age z-score < −2 SD from the 2006 WHO growth reference [435]), stunted (height-for-age z-score < −2 SD), and/or wasted (weight-for-height z- score < −2 SD).

Data analysis

We restricted this analysis to children who had at least one diarrhea episode and therefore had the opportunity to be treated with antibiotics for diarrhea. Because the proportion of missing data for baseline and diarrhea severity-related covariates was 5% or less for all variables, we imputed the median values of variables for individuals and episodes with missing data (indicated in Table 5.1 footnote).

Logistic regression was used to calculate inverse probability of exposure weights stabilized by the marginal probability of exposure [414]. Confounding variables for the exposure model were chosen by a causal DAG [415] to account for baseline characteristics and indications for treatment. We were particularly concerned about confounding by diarrhea episode severity, which was associated with higher antibiotic use rates and might also predict future diarrheal risk. We therefore included multiple characteristics of the diarrhea episode to capture the multifaceted concept of illness severity. The final exposure model included episode number, socioeconomic status defined from the modified Kuppuswamy scale [431,436], maternal education, child sex, Cesarean birth, low birth weight, preterm birth, hospitalization at birth, antibiotics given at birth, and characteristics of the last diarrhea episode: age, Vesikari score [412], duration, hospitalization, fever, dehydration, bloody diarrhea, underweight, stunted, wasted, exclusive and any breastfeeding, zinc given, number of previous antibiotic courses for any illnesses, number of sick days between episodes, and other antibiotics given between episodes. Continuous variables

were modeled flexibly with restricted quadratic splines [416], and covariate specifications were compared by Akaike's information criterion. To remove extreme weight values [417], weights were censored at the 0.5^{th} and 99.5^{th} percentiles by resetting the value of weights greater than the 99.5^{th} percentile and less than the 0.5^{th} percentile to the value of the 99.5^{th} and 0.5^{th} percentile respectively.

We estimated inverse probability-weighted Kaplan-Meier (KM) curves [414,418] for the time to next diarrhea episode comparing children who did and did not receive antibiotics for the previous episode. The time scale [418] was from 48 hours after the previous diarrhea episode to the incident day of the next episode. Children were censored at drop-out, death, or the end of follow-up at 3 years of age. We assumed person-time during which children were temporarily unreachable was missing at random and drop-out was non-informative given the small proportion of drop-outs (n=50, 11.5% overall; n=18, 4.1% between the first and second diarrhea episode). We assessed each episode pair separately and then collapsed across episodes.

We calculated the time difference and time ratio at 50% diarrhea-free survival, the median survival time, from the weighted KM curves. Confidence intervals were constructed by bootstrap [419] with 200 resamples at the level of the individual to account for clustering of episodes within children.

We also estimated hazard ratios comparing the same exposure groups using marginal structural Cox models [418] with the same inverse probability weights. These models were estimated by pooled logistic regression with adjustment for time using a restricted quadratic spline [416]. Correlation between outcomes from the same child was accounted for using generalized estimating equations with a robust variance estimator.

Effect measure modification

We assessed modification of the effect of antibiotics by age at exposure by stratification. We also considered the effect of specific antibiotics commonly given, trimethoprim/sulfamethoxazole (cotrimoxazole) and cephalosporins (97.4% of which were cefixime), by comparing children receiving each drug with children given no antibiotics.

Sensitivity analyses

To validate caregiver-report of antibiotic treatment, we repeated analyses with alternative definitions of antibiotic exposure. First, we restricted the exposed group to only those children whose caregivers reported the name of a confirmed antibiotic in the free-response section of the questionnaire. Second, we considered children exposed if either their caregiver reported antibiotics were given (by indicating yes/no) or if an antibiotic prescription was recorded in clinic records during the diarrhea episode. Finally, we considered children exposed only if a confirmed antibiotic name was reported or if a prescription was recorded in the clinic records.

To assess the impact of long episode duration contributing to shorter time between episodes, we repeated the main analyses excluding all episode pairs where the first episode lasted for more than 7 days (n=194, 8.6% total; n=42, 9.8% among first episodes).

To assess whether antibiotics were associated with the severity of subsequent diarrhea when another episode occurred, we estimated the effects of antibiotic treatment for the previous episode on the severity and duration of the next episode. In models weighted for the same covariates as in above analyses, we used inverse probability-weighted linear regression with the Vesikari score and number of days with diarrhea as continuous outcomes. We also estimated the

adjusted relative risk for a severe (Vesikari ≥ 11) and prolonged/persistent (≥7 days) next episode using inverse probability-weighted log-binomial regression.

Last, we compared the results from the main study with two earlier cohorts of children from the same study area [396,397]. The earlier cohorts lacked complete records of antibiotics given to treat non-diarrheal illnesses, and the type of antibiotics given for diarrhea were unknown. In addition, the most recent earlier study was a smaller quasi-experimental study, in which children were followed once-weekly for only 2 years and enrolled after birth if still exclusively breastfed [396]. Despite these limitations, we present the results from these cohorts for completeness.

Results

Almost all children in the birth cohort (434 of 497, 87.3%) had at least one diarrhea episode and were included in the analysis. Of these, 412, 393, and 384 children completed the first, second, and third study year of follow-up respectively (drop-out rate of 11.5%). Six children died during follow-up; two deaths were associated with diarrhea. Most children were of low socioeconomic status (n=282, 65%, Table 5.1) and approximately half had poor household hygiene (n=210, 48.4%). By six months of age, most children had stopped exclusive breastfeeding (n=370, 85.3%) and had their first episode of diarrhea (n=307, 70.7%). Children who received antibiotics were slightly more likely to be from households with poor hygiene. These children stopped all breastfeeding on average one month earlier, and had their first diarrhea episode at younger ages (Table 5.1).

The total accumulated follow-up was 1013.3 person-years, including 981.8 diarrhea-free person-years included as person-time at risk in analyses. Incidence of diarrhea was highest

around 6 months of age, with an incidence of 32.4 episodes per 100 person-months among children between 5 and 7 months of age (Figure 5.1).

A total of 2,295 diarrhea episodes were reported, of which 658 (28.9%) were treated with antibiotics. We excluded 16 diarrhea episodes (0.7%) due to missing antibiotic treatment information. More than half of children (n=268, 61.8%) reported at least one antibiotic course for diarrhea, and 154 (35.5%) reported two or more antibiotic courses for diarrhea in the first 3 years of life. Antibiotic treatment of diarrhea was associated with older age at the time of the episode and increased episode severity and duration (Table 5.2). The most common antibiotic given was cotrimoxazole, accounting for 50.3% of caregiver-reported antibiotics and 57.8% of antibiotics prescribed at the study clinic for diarrhea. Cefixime accounted for another 24.6% of caregiver-reported antibiotics and 34.5% of antibiotic prescriptions at the clinic. All other antibiotics, such as fluoroquinolones, penicillins, and macrolides, were reported for less than 5% of cases.

Effect on diarrhea incidence

Of 434 children experiencing a first diarrhea episode, we excluded 3 children with missing antibiotic treatment and one child who dropped out on the first day following their first episode. Among children who had a second diarrhea episode (n=375, 87.2%), the median time to second diarrhea episode was 10 weeks (IQR: 3, 20). The crude difference in median time to second diarrhea episode among children who were treated with antibiotics for their first episode (n=84) compared to children who were not treated (n=289) was 2 weeks (median time difference (MTD): -2, 95% CI: -8, 3). The crude hazard ratio from a Cox proportional hazards model was 1.15 (95% CI: 0.77, 1.72).

Figure 5.2A shows inverse probability of treatment-weighted KM curves for time to second diarrhea episode among children who were (n=93) and were not (n=337) treated with antibiotics for their first episode. Based on the weighted curves, children who received antibiotics for their first diarrhea episode had their second episode on average 8 weeks earlier (MTD: -8, 95% CI: -10, -3) or twice as soon (median time ratio (MTR): 0.50, 95% CI: 0.38, 0.79) as children who did not receive antibiotics (Table 5.3). In a Cox proportional hazards model weighted for the same covariates, the adjusted hazard ratio was 1.38 (95% CI: 1.05, 1.82).

The effect of antibiotic treatment of the second diarrhea episode on time to third diarrhea was similar, while effects in later episode pairs were smaller (Figure 5.2B-E, Table 5.3). The overall adjusted time difference and ratio when collapsing all episode pairs were -4 weeks (95% CI: -9, 0) and 0.71 (95% CI: 0.44, 0.96) respectively (Figure 5.2F, Table 5.3).

Effect measure modification

The effect of antibiotics on time to next diarrhea was greatest among children who were treated with antibiotics for diarrhea under 6 months of age compared to antibiotic treatment between 6 months and 1 year and after 1 year of age (Figure 5.3, Table 5.3). A shorter time to next diarrhea was observed for both cotrimoxazole (MTD: -1, 95% CI: -7, 2) and cephalosporins (MTD: -3, 95% CI: -9, 0) compared to no antibiotics, though the effect was smaller for cotrimoxazole (Figure 5.4, Table 5.4).

Sensitivity analyses

Results under alternative exposure definitions were consistent with the main analyses, though the effect size diminished as the definitions became less sensitive and more specific

(Figure 5.5, Table 5.4). When excluding all previous episodes with greater than 7 days duration, diarrhea-free survival curves were similar to main analyses, and time differences and ratios were slightly larger in magnitude (Figure 5.6, Table 5.5).

When subsequent diarrhea occurred, the average Vesikari score and duration of the second episode were slightly lower among children who were treated with antibiotics during their first episode compared to those who were not (Table 5.6). Correspondingly, the risks for a severe or prolonged/persistent second diarrhea episode were lower among these children. However, the absolute differences in severity and duration were small (less than one point on the Vesikari scale and less than one day, respectively) and imprecise since few episodes were severe (10.4%) or of long duration (11.5%). The results were consistent when restricting to episodes which occurred under 6 months of age and when including all episode pairs (Table 5.7).

To validate our findings, we analyzed data from two previous cohorts conducted at this site [395–397]. One study [396] was conducted from 2008-2011 and included 160 children with at least one diarrhea episode. Prevalence of antibiotic treatment of diarrhea was lower, at 6.4% (50 of 785 total episodes with antibiotic treatment information). The second study [395,397], conducted from 2002-2006, included 390 children who had at least one diarrhea episode. Of 1,812 diarrhea episodes with known antibiotic treatment, 27.7% (n=502) were treated with antibiotics. Information on antibiotic treatment for other illnesses was missing. The weighted KM curves including all episode pairs from these earlier studies were consistent with the results from the main study. Combining all three cohorts, children who were treated with antibiotics for their first diarrhea episode had their second episode 3 weeks (MTD: -3, 95% CI: -7, 1) or 20% (MTR: 0.80, 95% CI: 0.53, 1.07) earlier than children who were not treated with antibiotics (Figure 5.7).

Discussion

This study provides the first evidence that antibiotic treatment of diarrhea may shorten the time between episodes, especially among younger infants. These results are directly applicable to diarrhea treatment decisions since antibiotic treatment is not essential for most cases of diarrhea. Specifically, according to Integrated Management of Childhood Illness (IMCI) protocols [437], antibiotic treatment was likely not indicated for a majority of cases in this study since only few episodes (0.9%) were associated with bloody stools. While antibiotics are a well-known cause of AAD [229], we provide further support for a sustained impact of antibiotics on diarrheal risk. These results, which focus on antibiotic treatment of diarrhea specifically, are consistent with our recent work demonstrating that any antibiotic exposure early in life is associated with increased diarrheal rates [438].

Antibiotic treatment of diarrhea had the greatest impact on time to next episode during the first two diarrhea episodes. This difference in effect may be due to young age at earlier episodes and high overall antibiotic exposure by the time of later episodes. The substantial increases in magnitude of the adjusted effects compared to the crude effect are largely due to removing confounding by age. Because the microbiota is underdeveloped and more susceptible to disturbances during infancy, antibiotic exposures at the youngest ages may have the largest impact on the microbiota, and correspondingly on diarrheal risk [20,280]. In addition, because of the high rates of antibiotic use in this population, four-fifths (83%) of the population had prior exposure to antibiotics by the third diarrhea episode. We hypothesize that antibiotics for diarrhea are likely to have the largest impact when they represent a majority of total antibiotic exposures, which occurs at earlier episodes and younger ages.

The difference in effect on diarrheal risk between cotrimoxazole and cefixime may result from their different spectrums of activity. Cotrimoxazole is broad-spectrum, but notably does not affect anaerobes [439], which dominate the gut microbiota [272]. Conversely, anaerobes are sensitive to cefixime, and this drug is also more effective against Gram-negative bacteria (especially Enterobacteriaceae) common in the gut [439]. Correspondingly, diarrhea as a drug-related adverse event is more commonly reported for cefixime (15-20%) compared to cotrimoxazole (<1-10%) [439,440]. Similarly, cephalosporins are one of the predominant drug classes noted to cause AAD [13,236]. The activity of cefixime against intestinal anaerobes may result in greater disruption of the gut microbiota and increased susceptibility to diarrheal pathogens.

In the minority of diarrhea episodes of bacterial etiology and for which antibiotics may have been indicated, the reduction in time to subsequent diarrhea may alternatively have been due to a temporary benefit of antibiotics followed by the recrudescence of the causative and antibiotic-susceptible agents, resulting in a second diarrhea episode.

As in any observational study, there is the potential for bias due to uncontrolled confounding, including by local environmental factors associated with force of transmission and pathogen-specific effects on the microbiome. However, this cohort has the advantage of a detailed record of illness characteristics that were likely the main indications for treatment. This study was limited by potential misclassification of exposure due to caregiver-reported treatment information. However, we also incorporated antibiotic prescriptions from clinic records, which likely captured the majority of antibiotic exposures since the clinic was located in the study area and provided free care and medicines. There was concordance between caregiver-reported and antibiotic prescriptions for diarrhea: 78% of antibiotic prescriptions during diarrhea episodes

were associated with caregiver-reported antibiotic treatment. Further, our results were consistent when we used alternative definitions of antibiotic exposure in sensitivity analyses.

Because there were few severe illnesses in our cohort, we considered diarrhea incidence the main outcome of interest. Antibiotic treatment was associated with slightly lower severity and duration of subsequent diarrhea episodes, but the differences were small and imprecise. Antibiotic treatment of diarrhea may also have unintended consequences for other illnesses such as respiratory infections, and other potential effects should be taken into account when making treatment decisions.

By providing evidence that antibiotics may cause direct harm to children through an association with decreased time to future diarrhea episodes, these findings will counter a commonly held assumption among doctors and caregivers that even if antibiotics are not strictly indicated, “at least they can’t hurt ” [15]. While rational use of antibiotics has been advocated to reduce antimicrobial resistance at the population level, rational use might also decrease future diarrheal risk among treated patients.

Table 5.1. Demographic characteristics of 434 children with at least one diarrhea episode in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	0 antibiotics reported for diarrhea (n=166)	1+ antibiotics reported for diarrhea (n=268)
	No. (%) children	No. (%) children
Household characteristics		
Socioeconomic status*		
Low	114 (68.7)	168 (62.7)
Medium	50 (30.1)	94 (35.1)
High	2 (1.2)	6 (2.2)
Maternal education		
No formal education	67 (40.4)	93 (34.7)
Primary/middle school	52 (31.3)	97 (36.2)
Higher secondary school	42 (25.3)	69 (25.7)
College/polytechnic/professional school	5 (3.0)	9 (3.4)
Poor household hygiene†	75 (45.2)	149 (55.6)
Crowding		
High (>4 people/room)	52 (31.3)	78 (29.1)
Medium (3.1-4 people/room)	63 (38.0)	103 (38.4)
Low (≤ 3 people/room)	51 (30.7)	87 (32.5)
Child characteristics		
Sex of child		
Male	87 (52.4)	147 (54.9)
Female	79 (47.6)	121 (45.1)
Cesarean section	29 (17.5)	45 (16.8)
Low birth weight‡	33 (20.3)	43 (16.3)
Preterm birth‡	19 (11.7)	26 (9.9)
Baby kept in ICU at birth	9 (5.4)	23 (8.6)
Antibiotics at birth‡	3 (1.9)	8 (3.1)
Age at first diarrhea		
<6 months	103 (62.0)	204 (76.1)
6 months – 1 year	44 (26.5)	52 (19.4)
>1 year	19 (11.4)	12 (4.5)
Age (months) at stopping exclusive breastfeeding (mean, SD)	4.2 (2.0)	3.8 (2.1)
Age (months) at stopping all breastfeeding (mean, SD)	17.4 (8.7)	16.2 (8.3)

*Socioeconomic status categories defined from the modified Kuppuswamy scale [431,436]
†Poor household hygiene was based a score of less than 12 on a scale developed from an assessment of water, food, and personal hygiene [432]
‡7 missing values for low birth weight; 9 missing values for preterm birth; 13 missing values for antibiotics at birth; SD – standard deviation

Table 5.2. Characteristics of diarrhea episodes and their association with antibiotic treatment among 434 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	No. (%) total episodes (n=2279*)	No. (%) episodes treated with antibiotics (n=658)	Crude OR† (95% CI)
Age at episode			
< 6 mo.	589 (25.8)	110 (16.7)	1.
6 mo. – 1 year	701 (30.8)	213 (32.4)	1.90 (1.46, 2.47)
1 – 2 years	596 (26.2)	209 (31.8)	2.35 (1.80, 3.07)
2 – 3 years	393 (17.2)	126 (19.1)	2.05 (1.53, 2.76)
Severity (Vesikari score)			
Mild (2-5)	1125 (49.4)	235 (35.7)	1.
Moderate (6-10)	900 (39.5)	302 (45.9)	1.91 (1.57, 2.33)
Severe (11-15)	221 (9.7)	104 (15.8)	3.37 (2.49, 4.55)
Very severe (16-20)	33 (1.4)	17 (2.6)	4.02 (2.00, 8.08)
Duration (days)			
Acute (1-6)	2011 (88.2)	549 (83.4)	1.
Prolonged (7-13)	234 (10.3)	93 (14.1)	1.76 (1.33, 2.32)
Persistent (≥14)	34 (1.5)	16 (2.4)	2.37 (1.20, 4.67)
Bloody stools			
No	2231 (97.9)	634 (96.4)	1.
Yes	48 (2.1)	24 (3.7)	2.52 (1.42, 4.47)
Fever‡			
No	1990 (87.3)	518 (78.7)	1.
Yes	289 (12.7)	140 (21.3)	2.67 (2.08, 3.43)
Dehydration			
No	1652 (72.5)	410 (62.3)	1.
Yes	627 (27.5)	248 (37.7)	1.98 (1.63, 2.41)
Hospitalization			
No	2219 (97.4)	623 (94.7)	1.
Yes	60 (2.6)	35 (5.3)	3.59 (2.13, 6.04)

*Excludes 16 episodes for which antibiotic treatment was unknown.
†Odds ratio for antibiotic treatment of diarrhea episode by diarrhea characteristic
‡Caregiver-reported

Table 5.3. Estimated effect of antibiotic treatment for the previous diarrhea episode on time to next episode by episode pair and age at first episode among 430 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

Episode pair	Antibiotics for previous episode	No. of children	Median time difference (weeks; 95% CI)*	Median time ratio (95% CI)*	Hazard ratio† (95% CI)*
1st to 2nd	No	337	0.	1.	1.
	Yes	93	-8 (-10, -3)	0.50 (0.38, 0.79)	1.38 (1.05, 1.82)
2nd to 3rd	No	273	0.	1.	1.
	Yes	94	-7 (-11, 1)	0.46 (0.29, 1.10)	1.53 (1.05, 2.23)
3rd to 4th	No	234	0.	1.	1.
	Yes	75	1 (-11, 11)	1.07 (0.37, 1.90)	0.79 (0.54, 1.16)
4th to 5th	No	174	0.	1.	1.
	Yes	71	1 (-11, 12)	1.06 (0.40, 2.00)	1.70 (0.98, 2.97)
>5th	No	588	0.	1.	1.
	Yes	322	-3 (-7, 4)	0.79 (0.53, 1.33)	1.24 (0.93, 1.64)
All	No	1606	0.	1.	1.
	Yes	655	-4 (-9, 0)	0.71 (0.44, 0.96)	1.35 (1.11, 1.64)
Age at first episode					
< 6 mo.	No	472	0.	1.	1.
	Yes	108	-4 (-6, 0)	0.60 (0.40, 1.00)	1.72 (1.27, 2.32)
6 – 12 mo.	No	491	0.	1.	1.
	Yes	212	-4 (-9, 3)	0.76 (0.53, 1.22)	1.42 (1.14, 1.76)
≥ 12 mo.	No	643	0.	1.	1.
	Yes	335	-2 (-10, 6)	0.91 (0.55, 1.32)	1.12 (0.82, 1.54)

*Weighted for episode number, socioeconomic status [431,436], maternal education, child sex, Cesarean birth, low birth weight, preterm birth, hospitalization at birth, antibiotics given at birth, and characteristics of the last diarrhea episode: age, Vesikari score [412], duration, hospitalization, fever, dehydration, bloody diarrhea, underweight, stunted, wasted, exclusive and any breastfeeding, zinc given, number of previous antibiotic courses for any illnesses, number of sick days between episodes, and other antibiotics given between episodes. The mean weight was 1.01 with range 0.29-16.18; after censoring at the 0.05th and 99.5th percentiles, the mean was 0.99 with range 0.31-5.77.
CI – confidence interval
†Assumes proportional hazards

Table 5.4. Estimated effect of antibiotic treatment for the first diarrhea episode on time to second episode using alternative definitions for antibiotic treatment among 434 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

Antibiotic exposure definition	Antibiotics for previous episode	No. of children	Median time difference (weeks; 95% CI)*	Median time ratio (95% CI)*
Cotrimoxazole§	No	1417	0.	1.
	Yes	384	-1 (-7, 2)	0.92 (0.50, 1.17)
Cephalosporins§	No	1417	0.	1.
	Yes	180	-3 (-9, 0)	0.79 (0.43, 1.00)
Caregiver-report yes	No	337	0.	1.
	Yes	93	-8 (-10, -3)	0.50 (0.38, 0.79)
Caregiver-report antibiotic name	No	380‡	0.	1.
	Yes	53	-4 (-10, 2)	0.71 (0.41, 1.17)
Caregiver-report yes or prescription from clinic	No	302	0.	1.
	Yes	128	-2 (-7, 4)	0.86 (0.56, 1.33)
Caregiver-report antibiotic name or prescription from clinic	No	335	0.	1.
	Yes	98	1 (-7, 5)	1.08 (0.55, 1.40)

*Weighted for episode number, socioeconomic status, maternal education, child sex, Cesarean birth, low birth weight, preterm birth, hospitalization at birth, antibiotics given at birth, and characteristics of the last diarrhea episode: age, Vesikari score, duration, hospitalization, fever, dehydration, bloody diarrhea, underweight, stunted, wasted, exclusive and any breastfeeding, zinc given, number of previous antibiotic courses for any illnesses, number of sick days between episodes, and other antibiotics given between episodes; CI – confidence interval

†Assumes proportional hazards

‡Includes missing antibiotic use as not exposed

§Includes all episodes due to small sample size per episode pair

Table 5.5. Estimated effect of antibiotic treatment for the previous diarrhea episode on time to second episode excluding previous episodes >7 days duration among 434 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

Episode pair	Antibiotics for previous episode	No. of children	Median time difference (weeks; 95% CI)*	Median time ratio (95% CI)*
1st to 2nd	No	306	0.	1.
	Yes	82	-8 (-10, -2)	0.50 (0.38, 0.83)
2nd to 3rd	No	246	0.	1.
	Yes	81	-7 (-12, 2)	0.50 (0.25, 1.22)
All	No	1488	0.	1.
	Yes	579	-4 (-9, -1)	0.71 (0.44, 0.93)

*Weighted for episode number, socioeconomic status, maternal education, child sex, Cesarean birth, low birth weight, preterm birth, hospitalization at birth, antibiotics given at birth, and characteristics of the last diarrhea episode: age, Vesikari score, duration, hospitalization, fever, dehydration, bloody diarrhea, underweight, stunted, wasted, exclusive and any breastfeeding, zinc given, number of previous antibiotic courses for any illnesses, number of sick days between episodes, and other antibiotics given between episodes; CI – confidence interval

Table 5.6. Estimated effect of antibiotic treatment for the first diarrhea episode on the severity and duration of subsequent diarrhea among 373 children who had a second episode from Vellore, Tamil Nadu, 2009–2013.

	Second diarrhea episode				
	Mean Vesikari score (SD)	Severe episode* N (%)	Total	Vesikari score aβ† (95% CI)	Severe episode* aRR§ (95% CI)
First diarrhea episode					
No antibiotics	7.0 (3.3)	46 (15.9)	289	0.	1.
Antibiotics	7.0 (3.5)	14 (16.7)	84	-0.20 (-1.13, 0.73)	0.71 (0.37, 1.37)
	Mean duration (days; SD)	Prolonged/ persistent episode‡ N (%)	Total	Duration (days) aβ# (95% CI)	Prolonged/ persistent episode‡ aRR§ (95% CI)
No antibiotics	4.3 (3.7)	42 (14.5)	289	0.	1.
Antibiotics	3.4 (2.2)	10 (11.9)	84	-0.66 (-1.36, 0.03)	0.91 (0.41, 1.99)

*Vesikari score ≥ 11

†Difference in Vesikari score adjusted for episode number, socioeconomic status [431,436], maternal education, child sex, Cesarean birth, low birth weight, preterm birth, hospitalization at birth, antibiotics given at birth, and characteristics of the last diarrhea episode: age, Vesikari score [412], duration, hospitalization, fever, dehydration, bloody diarrhea, underweight, stunted, wasted, exclusive and any breastfeeding, zinc given, number of previous antibiotic courses for any illnesses, number of sick days between episodes, and other antibiotics given between episodes.

‡Duration ≥ 7 days

§Risk ratio adjusted for covariates listed in †

#Difference in diarrhea duration (days) adjusted for covariates listed in †

SD – Standard deviation; CI – confidence interval

Table 5.7. Estimated effect of antibiotic treatment for the previous diarrhea episode on the severity and duration of the next diarrhea episode among 430 children from Vellore, Tamil Nadu, 2009–2013. **A** – Restricting to children under 6 months of age at previous episode; **B** – Including all episode pairs.

A	Next diarrhea episode				
				Vesikari score	Severe diarrhea
Previous diarrhea episode	Mean Vesikari score (SD)	Severe episode* N (%)	Total	aβ† (95% CI)	aRR§ (95% CI)
No antibiotic	7.2 (3.2)	72 (16.4)	440	0.	1.
Antibiotic	7.4 (3.5)	18 (16.8)	107	-0.26 (-1.04, 0.49)	0.63 (0.35, 1.13)
				Duration (days)	Prolonged or persistent diarrhea
	Mean duration (days; SD)	Prolonged/ persistent episode‡ N (%)	Total	aβ# (95% CI)	aRR§ (95% CI)
No antibiotic	4.3 (3.4)	76 (17.3)	440	0.	1.
Antibiotic	3.9 (2.5)	19 (17.8)	107	-0.46 (-1.01, 0.06)	0.87 (0.50, 1.51)

B	Next diarrhea episode				
				Vesikari score	Severe diarrhea
Previous diarrhea episode	Mean Vesikari score (SD)	Severe episode* N (%)	Total	aβ† (95% CI)	aRR§ (95% CI)
No antibiotic	6.5 (2.9)	136 (10.5)	1308	0.	1.
Antibiotic	6.5 (3.1)	57 (10.4)	541	-0.26 (-0.58, 0.05)	0.78 (0.56, 1.08)
				Duration (days)	Prolonged or persistent diarrhea
	Mean duration (days; SD)	Prolonged/ persistent episode‡ N (%)	Total	aβ# (95% CI)	aRR§ (95% CI)
No antibiotic	3.8 (3.4)	165 (12.6)	1308	0.	1.
Antibiotic	3.3 (2.3)	47 (8.7)	541	-0.45 (-0.72, -0.18)	0.67 (0.46, 0.98)

*Vesikari score ≥ 11
†Difference in Vesikari score adjusted for same covariates as in Table 5.6
§Risk ratio adjusted for same covariates as in Table 5.6
‡Duration ≥ 7 days
#Difference in diarrhea duration (days) adjusted for same covariates as in Table 5.6
SD – Standard deviation; CI – confidence interval

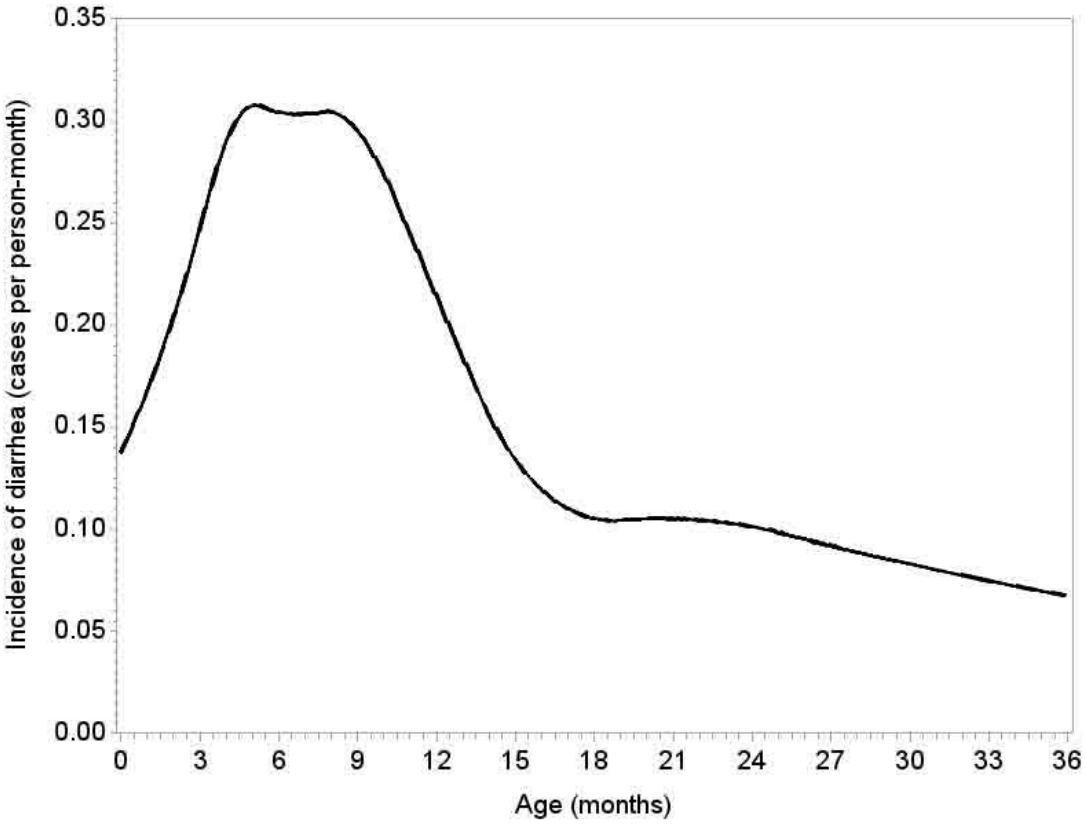

Figure 5.1. Incidence of diarrhea by age (using restricted quadratic splines [416]) among 434 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

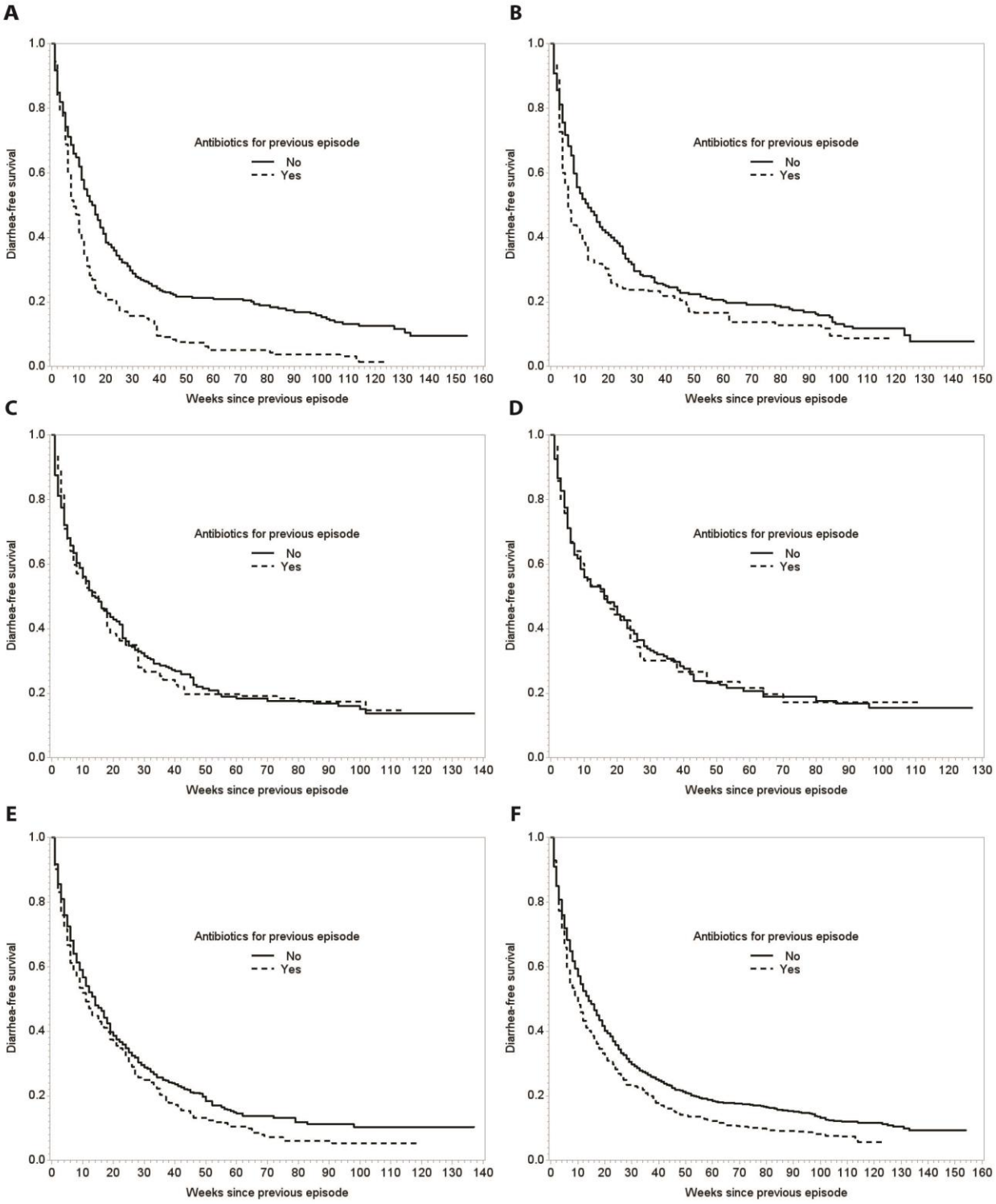

Figure 5.2. Inverse probability of treatment-weighted Kaplan-Meier curves for time to next diarrhea episode by antibiotic treatment for the previous diarrhea episode among 430 children from Vellore, Tamil Nadu, 2009–2013. Weighted diarrhea-free survival from: **A** – first to second episode; **B** – second to third episode; **C** – third to fourth episode; **D** – fourth to fifth episode; **E** – previous to next episode including episodes ≥6; **F** – all episode pairs.

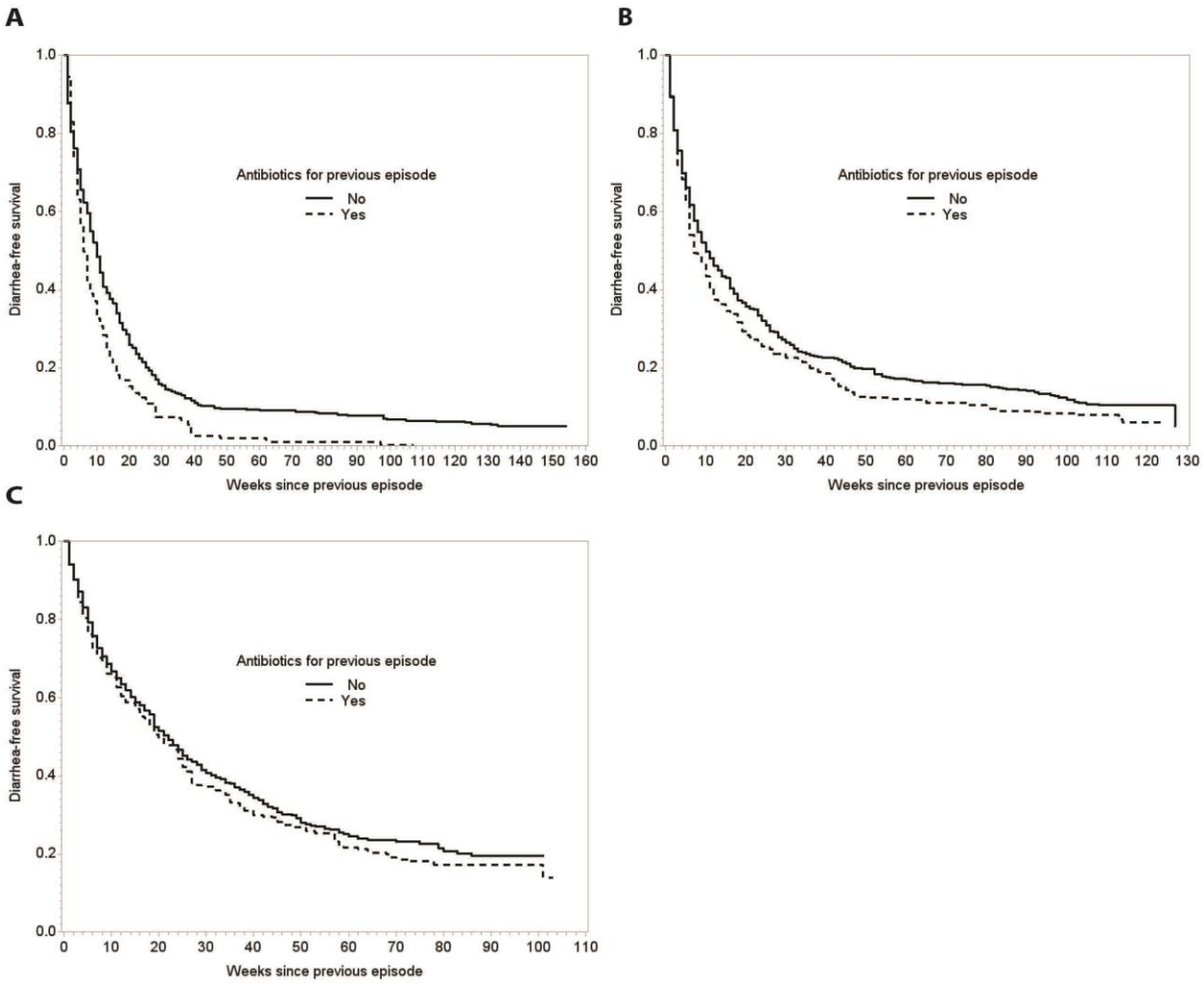

Figure 5.3. Stratified by age at first diarrhea episode, inverse probability of treatment-weighted Kaplan-Meier curves for time to next diarrhea episode among 430 children from Vellore, Tamil Nadu, 2009–2013. **A** – first diarrhea and antibiotic treatment below 6 months of age; **B** – first diarrhea and antibiotic treatment between 6 months and 1 year of age; **C** – first diarrhea and antibiotic treatment after 1 year of age.

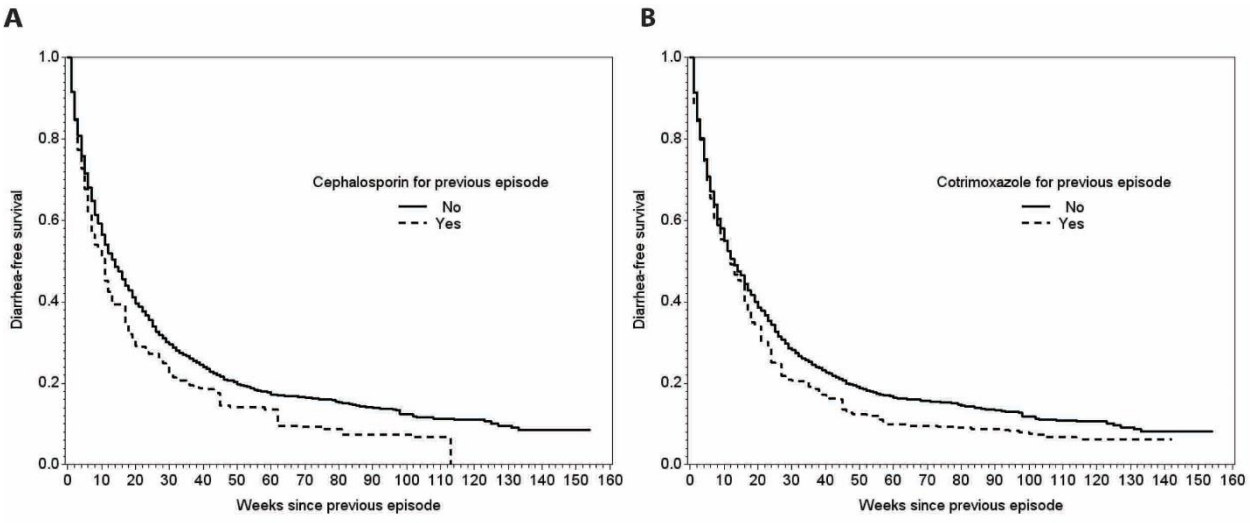

Figure 5.4. Stratified by antibiotic type given for the previous diarrhea episode, inverse probability of treatment-weighted Kaplan-Meier curves for time to next diarrhea episode among 430 children from Vellore, Tamil Nadu, 2009–2013. **A** – Antibiotic treatment with a cephalosporin versus no antibiotics; **B** – Antibiotic treatment with cotrimoxazole versus no antibiotics.

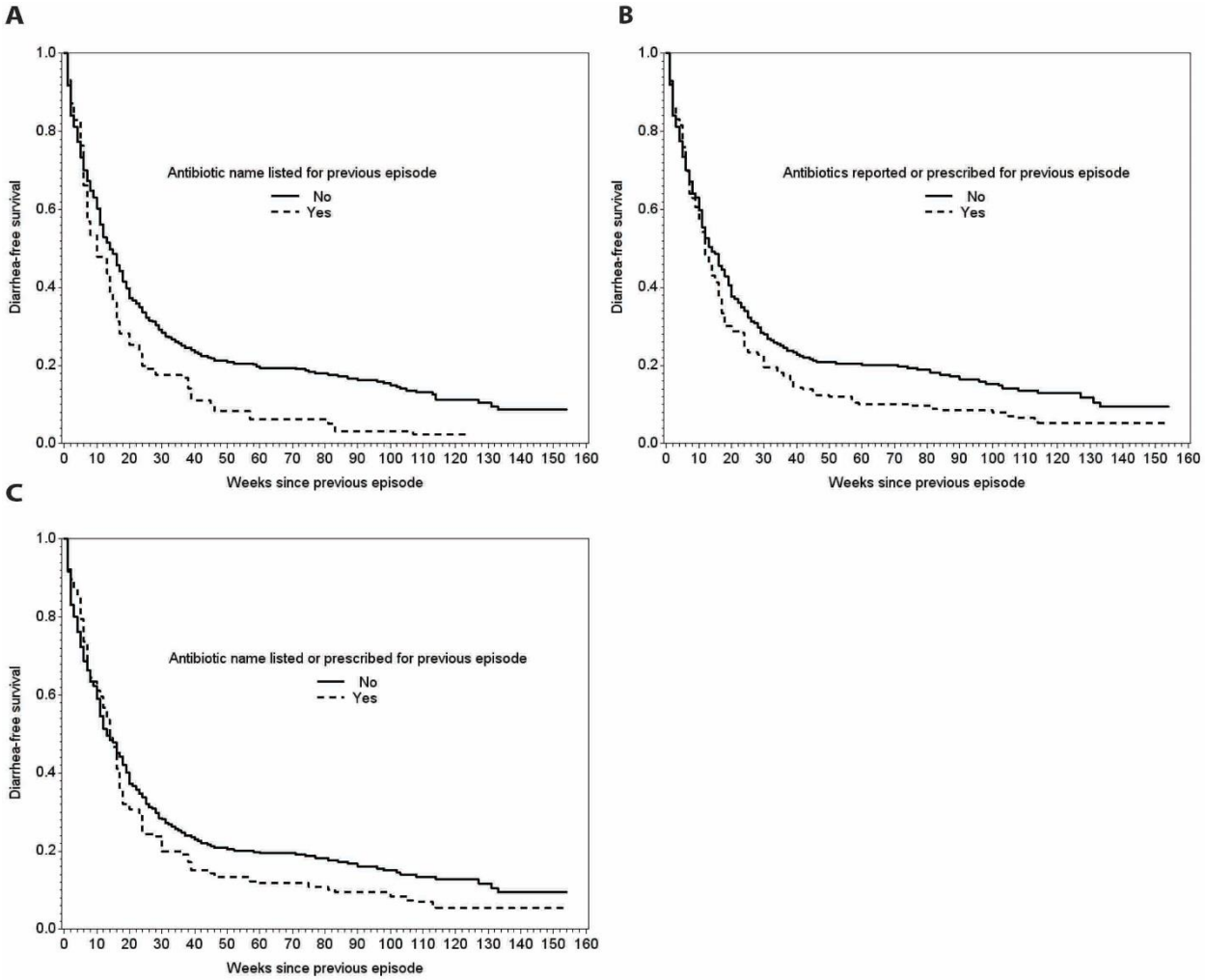

Figure 5.5. Inverse probability of treatment weighted Kaplan-Meier curves for time to second diarrhea episode using alternative definitions for antibiotic treatment of the first diarrhea episode among 430 children from Vellore, Tamil Nadu, 2009–2013. **A** –Children exposed only if caregivers reported the name of an antibiotic; **B** – Children exposed if either their caregiver reported antibiotics were given or if an antibiotic prescription was recorded in clinic records during the diarrhea episode; **C** – Children exposed if an antibiotic was listed in the free-response section of the questionnaire or if a prescription was recorded in the clinic records.

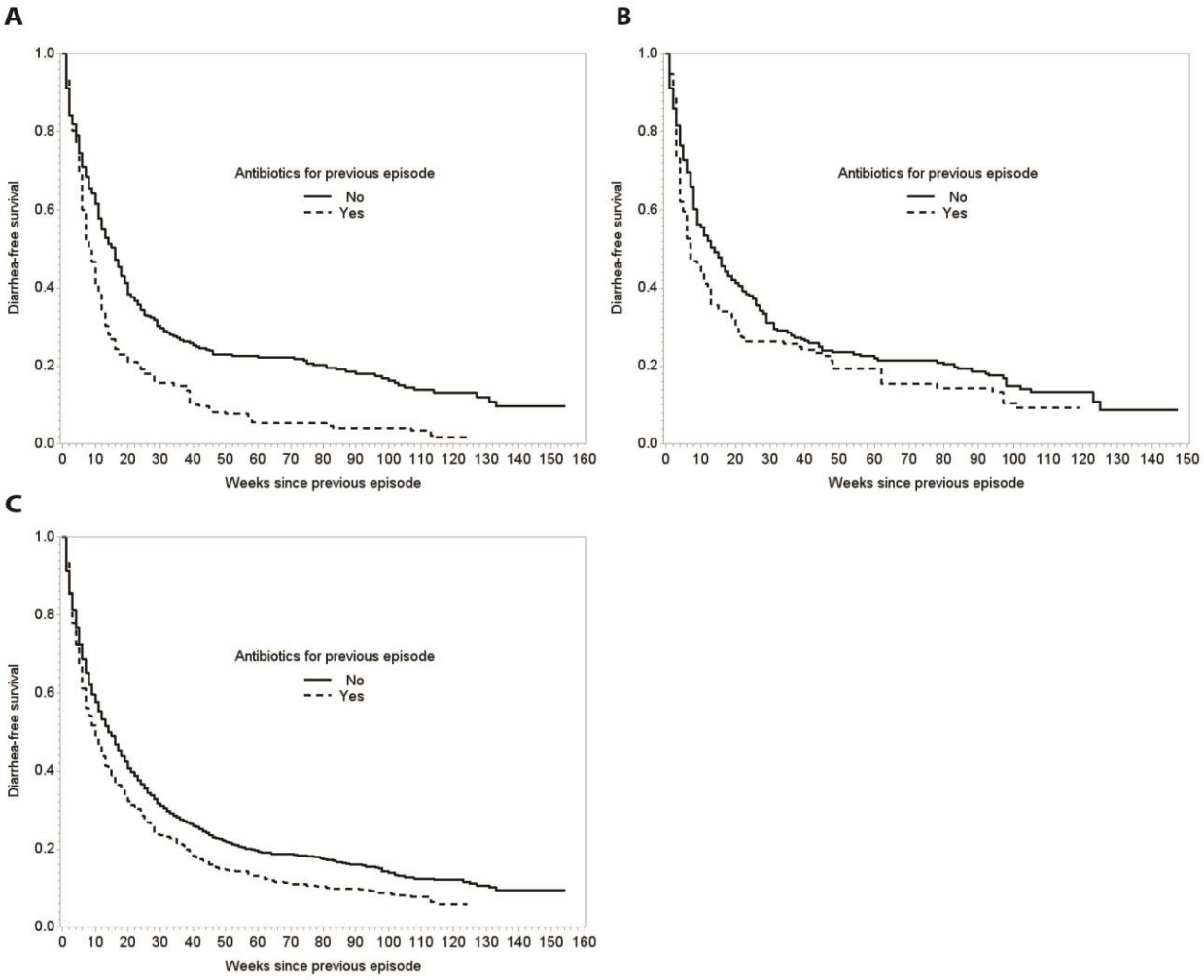

Figure 5.6. Inverse probability of treatment-weighted Kaplan-Meier curves for time to next diarrhea episode by antibiotic treatment for the previous diarrhea episode excluding previous episodes >7 days duration among 430 children from Vellore, Tamil Nadu, 2009–2013. **A** – Weighted diarrhea-free survival from first to second episode; **B** – Weighted diarrhea-free survival from second to third episode; **C** – Weighted diarrhea-free survival for all episode pairs.

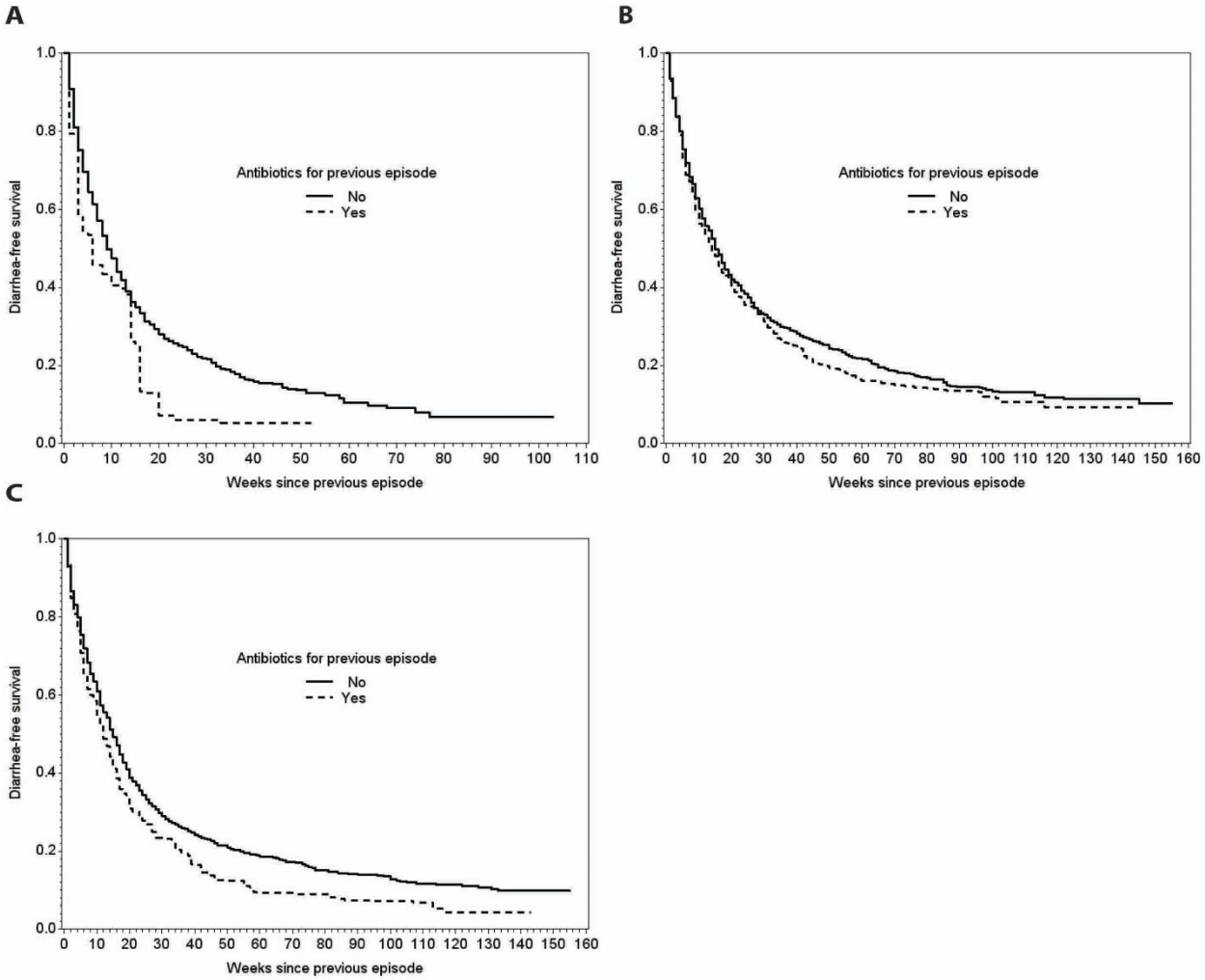

Figure 5.7. Inverse probability of treatment-weighted Kaplan-Meier curves for time to next diarrhea episode by antibiotic treatment for the previous diarrhea episode in alternative cohorts. **A** – All episodes among a cohort of 160 children from Vellore, Tamil Nadu, 2008–2011; **B** – All episodes among a cohort of 390 children from Vellore, Tamil Nadu, 2002–2006; **C** – From first to second episode among all three cohorts of 390 (2002-2006), 160 (2008-2011), and 430 (2009-2013) children from Vellore, Tamil Nadu.

CHAPTER VI: THE EFFECT OF EARLY LIFE ANTIBIOTIC EXPOSURES ON DIARRHEAL RATES AMONG YOUNG CHILDREN IN VELLORE, INDIA

Abstract

Background

Antibiotic treatment of childhood illnesses is common in India. In addition to contributing to antimicrobial resistance, antibiotics may result in increased susceptibility to diarrhea through interactions with the gastrointestinal microbiota. Breast milk, which enriches the microbiota early in life, may increase the resilience of the microbiota against perturbations by antibiotics.

Methods

In a prospective observational cohort study, we assessed whether antibiotic exposures from 0-5 months affected rates of diarrhea up to age 3 among 465 children from Vellore, India. Adjusting for treatment indicators, we modeled diarrheal rates among children exposed and unexposed to antibiotics using negative binomial regression. We further assessed whether the effect of antibiotics on diarrheal rates was modified by exclusive breastfeeding at 6 months.

Results

More than half of children (n=267, 57.4%) were given at least one course of antibiotics in the first 6 months of life. The adjusted relative incidence rate of diarrhea was 33% higher among children who received antibiotics under 6 months of age compared to those who did not

(incidence rate ratio: 1.33, 95% confidence interval: 1.12, 1.57). Children who were exclusively breastfed until 6 months of age did not have increased diarrheal rates following antibiotic use.

Conclusions

Antibiotic exposures early in life were associated with increased rates of diarrhea in early childhood, but exclusive breastfeeding may protect against this negative impact. While antibiotics must be used for treatment when necessary, the potential for increased susceptibility to diarrhea should be further explored.

Introduction

Antibiotics are frequently used to treat childhood illnesses, especially respiratory tract infections, otitis media, and diarrhea. However, many of these infections are viral and/or self-limiting, such that antibiotics are not necessary [189,191,213]. The overuse of antibiotics among children has been reported around the world [213,219,220], and antibiotic over-prescribing by primary care physicians [9,208] in India is further compounded by the ability to purchase antibiotics over the counter without a prescription [210] in spite of government regulations [209].

Often, the primary rationale given for restricting antibiotic use is to slow the development of antimicrobial resistance [15,211]. However, recent evidence has suggested antibiotics may also have a direct negative impact on the patients prescribed the drugs, primarily through interactions with the GI microbiota [15]. The development of the GI microbiota in the first few months of life coincides with a critical period of intestinal structure and immune system maturation [265,279,303]. A healthy microbiota is important in the early life defense against gastrointestinal infections by providing a barrier effect that inhibits the attachment and growth of pathogens [433,434]. Antibiotics impact the diversity and composition of the GI microbiota, and

some of these effects can persist long after treatment is completed [17,351], especially among infants [20,251,280,291,340]. These exposures have been further associated with impaired GI functioning, intestinal inflammation, increased intestinal permeability, and increased risk of systemic infections [301,340,357].

Given the potential negative impact of antibiotics on the developing microbiota, we assessed whether antibiotic exposure in the first 6 months of life affected subsequent rates of all-cause diarrhea from 6 months to 3 years of age in a birth cohort from Vellore, India. We also explored the impact of exclusive breastfeeding, which may modify this effect given the beneficial role of breast milk on the microbiota [288,290,441].

Methods

We performed an analysis of data from a prospective observational cohort study of immune responses in cryptosporidiosis in 497 children followed from birth to 3 years of age. The parent study population consisted of all children born in four geographically adjacent, semi-urban slums of Vellore, in the state of Tamil Nadu, India between April 2009 and May 2010. The study population, enrollment strategy, and data collection methods have been previously described [28]. The study was approved by the Institutional Review Boards of the Christian Medical College, Vellore, India, Tufts University Health Sciences campus, Boston, USA, and University of North Carolina – Chapel Hill, USA.

Briefly, baseline information on maternal demographic characteristics, socioeconomic indicators, health-seeking behavior, environment, diet, and characteristics of delivery were collected within 45 days of birth. Fieldworkers visited households of all enrolled children twice per week until 3 years of age to collect information on incidence, duration, and hospitalization

for all illnesses as well as specific severity and treatment details for reported diarrhea. Caregivers were asked specifically if any antibiotics were given for diarrhea (yes/no) and if so, to report the name of the antibiotic (available for 64.0% of antibiotic reports). Breastfeeding history (exclusive, non-exclusive, none) was collected every two weeks until breastfeeding was stopped completely. Height and weight were measured monthly at the study clinic. Antibiotic prescriptions and corresponding diagnoses were also extracted from records at the physician-run study clinic that was established in the study area to provide free health care to study children.

Data and definitions

The main exposures were any antibiotic exposure in the first 6 months of life, as well as the total number of antibiotic courses in the first 6 months of life, both based on a combination of antibiotic prescriptions recorded in clinic records and caregiver-reported antibiotic treatment at birth and for diarrhea. We excluded all topical antibiotics (neosporin, neomycin, soframycin, and gentian violet). This clinical definition of antibiotic exposure was used in all primary analyses. Exclusive breastfeeding was defined according to the standard WHO definition [411] as feeding with breast milk only with the exception of vitamins, mineral supplements, and medicines (no liquid, semisolid, or solid food).

Diarrhea outcomes were based on caregiver-reported diarrhea at twice-weekly home visits. Diarrhea was defined using the standard WHO definition as at least three loose or watery stools in a 24-hour period [10]. A new episode was defined as diarrhea that occurred after at least 2 days of normal bowel movements. Rates of diarrhea after 6 months of age per child were defined by the total number of incident episodes divided by the total time that child remained in the study. We excluded from person-time denominators days with diarrhea (when a child was not

at risk of incident diarrhea, 0.3% of total person-time from 6 months to 3 years), periods during which the child was unreachable (0.6% of person-time from 6 months to 3 years), and any time after loss to follow-up or death (8.2% of person-time). Because the proportion of missing data was less than 3% for all baseline variables (see footnote in Table 6.1), we imputed the median values of variables for individuals with missing data.

Data analysis

We used Poisson and negative binomial regression to model the rates of diarrhea from 6 months to 3 years of age. The negative binomial model was preferred over the Poisson model by likelihood ratio test ($p<0.0001$) and was used in final analyses to correct for over-dispersion [442]. Crude and adjusted incidence rate ratios (IRRs) for diarrhea were estimated comparing children who were exposed to early life antibiotics to those who were not. Confounding variables were chosen by a causal DAG [415] based on the substantive literature, and optimal variable coding was determined by likelihood ratio test ($\alpha=0.1$) and Akaike's information criterion (AIC).

Using the causal DAG, we identified the following demographic characteristics and measures of illness in the first 6 months for adjustment in the models: child sex, socioeconomic status based on the Kuppuswamy scale [431]), maternal education, household hygiene [432], household crowding, low birth weight (<2.5 kg), number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score [412] of any diarrhea episode in first 6 months, number of severe episodes in first 6 months (Vesikari ≥ 11), prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight (average weight-for-age z-score under 6 months of age <-2 SD from the 2006

WHO growth reference [435]), stunting (average height-for-age z-score <−2 SD), and wasting (average weight-for-height z-score <−2 SD) in the first 6 months, any severe illness in first 6 months, and number of other infections in first 6 months.

Effect measure modification

We assessed whether the effect of early life antibiotic exposure on diarrheal risk after 6 months was modified by exclusive breastfeeding at 6 months of age or at first antibiotic exposure by reporting stratum-specific estimates and testing homogeneity by likelihood ratio test (α=0.1). We further explored the role of breastfeeding by assessing the crude association between exclusive breastfeeding and antibiotic treatment (both any exposure under six months, using log-risk regression, and age at first exposure, using log-transformed age with linear regression). We further assessed effect measure modification by sex, Cesarean section birth, age at first diarrhea, and growth status (underweight, stunted, wasted) in first 6 months.

Sensitivity analyses

To assess potential misclassification of the exposure, we repeated main analyses with more restricted definitions of antibiotic exposure that included caregiver-reported antibiotics only if an antibiotic name was recorded. To determine if the effect of antibiotics on diarrheal rates differed by antibiotic type [443,444], we repeated analyses separately comparing children who exclusively received one of the most commonly used antibiotics, amoxicillin and cotrimoxazole, to children who received no antibiotics. We were unable to assess other major classes of antibiotics due to few children receiving other drugs. We further assessed if the effect of antibiotics differed depending on 1) the indication for which antibiotic treatment was given; 2)

the number of diarrhea episodes experienced in the first 6 months of life; and 3) the time period for diarrheal outcomes (6-18 months of age compared to 18-36 months).

Results

We included 465 of 497 children in the parent cohort (93.6%) who remained in the study for more than 6 months and were therefore at risk for diarrhea after 6 months of age. An additional 11.8% of children were lost to follow-up over the remaining study period; 21, 24, and 10 children dropped out before 1, 2, and 3 years of age respectively. All person-time during which these children remained in the study was included in the analysis, and differential follow-up times were accounted for in the analytic models. Six children died after 6 months of age, and two of these deaths were associated with diarrhea. Two-thirds of children were of low socioeconomic status (n=308, 66.2%, Table 6.1). Children living in crowded households with more than 4 people per room were common (n=149, 32.0%), and approximately half of mothers reported poor household hygiene (n=221, 47.5%). By six months of age, most children had stopped exclusive breastfeeding (n=394, 84.7%) and had their first episode of diarrhea (n=300, 64.5%). Demographic characteristics of the children by exclusive breastfeeding to at least 6 months of age are shown in Table 6.2.

Rates of diarrhea were highest between 3 and 9 months of life, with crude incidence rates of 28.1 cases per 100 person-months among children aged 3-6 months and 30.3 cases per 100 person-months among children aged 6-9 months. After 6 months of age, diarrhea rates decreased to an average rate of 13.4 cases per 100 person-months from 6 months to 3 years, corresponding to a total of 1,693 episodes, or an average of 3.6 episodes per child in that period. The median number of diarrhea episodes after 6 months of age was 2 (interquartile range: 1, 5; Figure 6.1).

Antibiotic exposure in the first 6 months of life was common. More than half (n=267, 57.4%) of children were given at least one course of antibiotics in the first 6 months (Table 6.1). At the study clinic, antibiotic prescriptions in this age group were most frequently associated with diagnoses for upper respiratory infections (n=160, 36.5% of total prescriptions), acute gastroenteritis (including diarrhea; n=111, 25.3%), lower respiratory infections (n=71, 16.2%), and acute otitis media (n=18, 4.1%). Almost all children (n=415, 89.2%) also received antibiotics after 6 months of age, and children who were given antibiotics under 6 months were more likely to receive antibiotics after 6 months compared to children who were not given antibiotics under 6 months (Table 6.1).

Amoxicillin and sulfamethoxazole/trimethoprim (cotrimoxazole) were the most commonly given antibiotics. Children were generally given amoxicillin for respiratory illnesses, and cotrimoxazole for gastrointestinal illnesses. Approximately one-third of children received either or both of these antibiotics before 6 months of age. Less common antibiotic exposures were cefixime (4.3% of children received cefixime under 6 months of age), fluoroquinolones (ciprofloxacin, norfloxacin, and levofloxacin; 2.4%), and azithromycin (1.7%).

Effect of antibiotics on diarrheal rates

Antibiotic exposure under 6 months of age was crudely associated with an increase in diarrhea rates from 6 months to 3 years (Table 6.3). After multivariable adjustment, the relative incidence rate of diarrhea was 33% higher among children who had at least one course of antibiotics under 6 months of age compared to those who did not (IRR: 1.33, 95% CI: 1.12, 1.57). There was no significant difference in the effect by the number of antibiotic courses

received. Children who received three or more courses of antibiotics had a slightly lower relative increase compared to one or two courses, though this estimate was less precise (Table 6.3).

Effect measure modification

The effect of early antibiotic exposure differed by exclusive breastfeeding practices in the first 6 months of life (*p* for heterogeneity = 0.003). Children who were exclusively breastfed until at least 6 months of age (n=71) did not show an increase in diarrheal rates associated with antibiotic exposure (Table 6.4). Conversely, among children who had discontinued exclusive breastfeeding before 6 months (n=394), any early antibiotic exposure was associated with a 48% relative increase in diarrheal rates (IRR: 1.48, 95% CI: 1.23, 1.78). Results were qualitatively similar when restricting to exclusive breastfeeding at the first antibiotic exposure: the relative increase in diarrheal rates among children who were exclusively breastfed at their first antibiotic exposure was less than that among children who were not exclusively breastfed at their first antibiotic exposure (Table 6.5).

While breastfeeding modified the effect of antibiotics, duration of exclusive breastfeeding was not crudely associated with antibiotic use in the first 6 months of life. Children who were exclusively breastfed until at least 6 months were equally likely to be exposed to early life antibiotics as children who stopped exclusive breastfeeding before 6 months (crude risk ratio: 0.94, 95% CI: 0.76, 1.15). There was also no crude linear relationship between age at stopping exclusive breastfeeding and age at first antibiotic use (β = -0.0000, 95% CI: -0.0013, 0.0012).

There was no evidence of effect measure modification of the impact of antibiotics on diarrheal rate by sex, Cesarean section birth, age at first diarrhea, or growth status in the first 6 months of life.

Sensitivity analyses

In an alternative definition of antibiotic exposure, we included caregiver-report only if an antibiotic was named. Adjusted effects were qualitatively similar, but slightly closer to the null, for both overall effects and effects stratified by exclusive breastfeeding (Table 6.6).

There were no differences in the effect on diarrheal rates by antibiotic drug type. A sufficient number of children received amoxicillin (n=145) and cotrimoxazole (n=158) for multivariate analyses. The adjusted IRR comparing children exposed to only amoxicillin to children who received no antibiotics was 1.46 (95% CI: 1.12, 1.90), which was similar to the rate ratio comparing children exposed to only cotrimoxazole to unexposed children: 1.32 (95% CI: 1.04, 1.69; Table 6.7).

There was no difference in the effect of antibiotics on diarrheal rates depending on the indicating illness for antibiotic treatment. Children treated with antibiotics for only diarrheal and only non-diarrheal illnesses had similar increases in diarrhea rates after 6 months of age (Table 6.8). The effect was also the same regardless of the number of diarrhea episodes experienced during the first 6 months (Table 6.9), and the time period for diarrheal outcomes (6-18 months of age compared to 18-36 months; Table 6.10).

Discussion

Our study provides the first evidence that antibiotic treatment early in life is associated with increased rates of all-cause diarrhea from 6 months to 3 years of age, even when controlling for indications for treatment such as illness burden and severity. Further, we found that exclusive breastfeeding during antibiotic exposure may be protective against the effects of antibiotics since antibiotic exposure was not associated with increased diarrheal rates among children who were exclusively breastfed for at least the first 6 months of life.

The effect of antibiotic treatment on diarrheal risk may be mediated by a prolonged effect of the antibiotics on microbiota composition [17] or through collateral effects on intestinal structure and function relating to inflammation, permeability, and intestinal immunity [247,357]. In this way, antibiotics could affect susceptibility to diarrhea due to diverse causes. The strong effect modification by exclusive breastfeeding may also be explained by interactions with the microbiota. Bacteria such as *Lactobacillus* are present in breast milk and are thought to be beneficial: in a randomized trial of a *Lactobacillus* strain present in breast milk, infants given the probiotic demonstrated reduced incidence of gastrointestinal infections [441,445]. The protective effect of breastfeeding suggests the microbiota enriched by breast milk may be more resistant to perturbations by antibiotics.

Antibiotic treatment in the first 6 months of life was common among study children (57.4%). In the US and UK, the proportion of children treated under 6 months of age based on caregiver-report is reported to be lower, at approximately one third of children [11,12]. Higher rates of antibiotic use in our study population may be due to higher rates of infection, better capture of antibiotic prescriptions in clinic records, and greater availability of antibiotics without prescriptions.

The main limitation of this study was potential under-ascertainment of antibiotic exposures for antibiotics obtained outside of the study clinic for non-diarrheal illnesses. However, we expect almost all antibiotic exposures to be captured in clinic records since the study clinic was conveniently located in the residential area where study children lived and provided clinical care and medicines free of charge. High concordance between caregiver-reported and antibiotic prescriptions for diarrhea supports our assumption that most antibiotic exposures were recorded in clinic records (78% of antibiotic prescriptions during diarrhea episodes were associated with caregiver-reported antibiotic treatment). The validity of caregiver-reported antibiotics was supported in sensitivity analyses by consistent results when using a more restricted definition of antibiotic exposure that required caregivers to list an antibiotic name.

We also did not have information on the duration of antibiotic exposures. Length of therapy may be an important component of the effect of antibiotics on diarrheal risk given the potential for longer antibiotic exposure periods to have increased impact on the microbiota [17,351,443]. While we were unable to assess the potential dose-response relationship between duration of exposures and increased diarrheal rates, we do not expect that this more detailed information would alter our overall conclusions of an effect of antibiotics on diarrheal rates.

While we cannot ensure the absence of confounding in this observational study, we have adjusted for detailed treatment indications associated with disease severity that also predict diarrheal risk. As well, because a clinical trial which randomized all antibiotic treatment in this setting would be unethical, we believe this evidence from a well-conducted prospective observational cohort study with good follow-up provides important preliminary evidence towards understanding the impact of antibiotics on diarrheal risk. Antibiotics are commonly given during infancy at a time in which the developing gastrointestinal microbiota is most

sensitive to perturbations. While these drugs are lifesaving and should be used for treatment when necessary, the possibility for antibiotics to increase risk of future diarrheal disease should be further explored and potentially considered when making treatment decisions.

Table 6.1. Demographic characteristics of 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	No antibiotics 0-5 months (n=198)	Antibiotics 0-5 months (n=267)
	No. children (%)	No. children (%)
Household characteristics		
Socioeconomic status*		
Low	139 (70.2)	169 (63.3)
Medium	56 (28.3)	93 (34.8)
High	3 (1.5)	5 (1.9)
Maternal education		
No formal education	71 (35.9)	99 (37.1)
Primary/middle school	72 (36.4)	86 (32.2)
Higher secondary school	50 (25.3)	73 (27.3)
College/polytechnic/professional school	5 (2.5)	9 (3.4)
Poor household hygiene†	89 (45.0)	135 (50.6)
Crowding		
Low (≤ 3 people/room)	39 (19.7)	96 (36.0)
Medium (3.1-4 people/room)	90 (45.5)	91 (34.1)
High (>4 people/room)	69 (34.9)	80 (30.0)
Child characteristics		
Sex of child		
Male	97 (49.0)	149 (55.8)
Female	101 (51.0)	118 (44.2)
Cesarean section	32 (16.2)	50 (18.7)
Low birth weight‡	36 (18.2)	42 (15.7)
Baby kept in ICU at birth	10 (5.1)	23 (8.6)
Antibiotics at birth‡	0	12 (4.5)
Age at first diarrhea		
<6 months	100 (50.5)	200 (74.9)
6 months – 1 year	58 (29.3)	38 (14.2)
>1 year	17 (8.6)	14 (5.2)
No diarrhea	23 (11.6)	15 (5.6)
Number of diarrhea episodes 0-5 months		
0	98 (49.5)	67 (25.1)
1	52 (26.3)	75 (28.1)
2	34 (17.2)	68 (25.5)
3+	14 (7.1)	57 (21.4)
Age (months) at stopping exclusive breastfeeding (mean, SD)	4.0 (2.20)	3.9 (1.98)
Age (months) at stopping all breastfeeding (mean, SD)	16.8 (7.95)	16.7 (8.57)
Underweight in first 6 mo.	50 (25.3)	74 (27.7)
Stunted in first 6 mo.	34 (17.2)	67 (25.1)

Wasted in first 6 mo.	34 (17.2)	38 (14.2)
Antibiotic use in first 6 mo.		
1	–	135 (50.6)
2	–	74 (27.7)
3+	–	58 (21.7)
Antibiotic use 6 mo.-3 years.		
1	23 (11.6)	16 (6.0)
2	27 (13.6)	22 (8.2)
3+	118 (59.6)	209 (78.3)

*Socioeconomic status categories defined from the Kuppuswamy scale based on educational and occupational level of the family, house ownership, total number of rooms in the house, and household possessions [431]

†Poor household hygiene was based on a score of less than 12 on a scale developed from an assessment of water, food, and personal hygiene [432]

‡7 missing values for low birth weight; 14 missing values for antibiotics at birth; SD – standard deviation; ICU – intensive care unit

Table 6.2. Demographic characteristics of 465 children by exclusive breastfeeding at 6 months of age in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	Exclusively breastfed until at least 6 mo. (n=71)	No longer exclusively breastfed at 6 mo. (n=394)
	No. children (%)	No. children (%)
Household characteristics		
Socioeconomic status*		
Low	53 (74.7)	255 (64.7)
Medium	17 (23.9)	132 (33.5)
High	1 (1.4)	7 (1.8)
Maternal education		
No formal education	30 (42.3)	140 (35.5)
Primary/middle school	23 (32.4)	135 (34.3)
Higher secondary school	17 (23.9)	106 (26.9)
College/polytechnic/professional school	1 (1.4)	13 (3.3)
Poor household hygiene†	36 (50.7)	208 (52.8)
Crowding		
Low (≤ 3 people/room)	19 (26.8)	116 (29.4)
Medium (3.1-4 people/room)	27 (38.0)	154 (39.1)
High (>4 people/room)	25 (35.2)	124 (31.5)
Child characteristics		
Sex of child		
Male	39 (54.9)	207 (52.5)
Female	32 (45.1)	187 (47.5)
Cesarean section	10 (14.1)	72 (18.3)
Low birth weight‡	11 (15.5)	67 (17.0)
Baby kept in ICU at birth	4 (5.6)	29 (7.4)
Antibiotics at birth‡	3 (4.2)	9 (2.3)
Age at first diarrhea		
<6 months	41 (57.8)	259 (65.7)
6 months – 1 year	17 (23.9)	79 (20.1)
>1 year	6 (8.5)	25 (6.4)
No diarrhea	7 (9.9)	31 (7.9)
Number of diarrhea episodes 0-5 months		
0	30 (42.3)	135 (34.3)
1	17 (23.9)	110 (27.9)
2	18 (25.4)	84 (21.3)
3+	6 (8.5)	65 (16.5)
Age (months) at stopping exclusive breastfeeding (mean, SD)	7.0 (1.34)	3.4 (1.67)
Age (months) at stopping all breastfeeding (mean, SD)	19.9 (7.45)	16.2 (8.33)
Antibiotic use in first 6 mo.		
0	28 (39.4)	170 (43.2)

1	20 (28.2)	115 (29.2)
2	17 (23.9)	57 (14.5)
3+	6 (8.5)	52 (13.2)

*Socioeconomic status categories defined from the Kuppuswamy scale based on educational and occupational level of the family, house ownership, total number of rooms in the house, and household possessions

†Poor household hygiene was based on a score of less than 12 on a scale developed from an assessment of water, food, and personal hygiene

‡7 missing values for low birth weight; 14 missing values for antibiotics at birth; SD – standard deviation

Table 6.3. Estimated effect of antibiotic exposure in the first 6 months of life on incident rates of diarrhea from 6 months to 3 years of age among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

				Incidence rate ratio (95% CI)	
		No. of children	Rate of diarrhea*	Crude	Adjusted†
Antibiotics <6 months	No	198	10.1	1.	1.
	Yes	267	15.9	1.58 (1.33, 1.88)	1.33 (1.12, 1.57)
Number of antibiotic courses	0	198	10.1	1.	1.
	1	135	15.0	1.49 (1.22, 1.83)	1.36 (1.12, 1.64)
	2	74	16.7	1.66 (1.30, 2.11)	1.35 (1.07, 1.70)
	3+	58	17.0	1.69 (1.28, 2.22)	1.17 (0.89, 1.55)

*Rate per 100 person-months

†Incident rate ratio adjusted for covariates listed in the methods and specified in the supplemental material.

Table 6.4. Estimated effect of antibiotic exposure in the first 6 months of life on rates of diarrhea from 6 months to 3 years of age by exclusive breastfeeding at 6 months of age among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

		No. of children	Adjusted IRR* (95% CI)
Exclusively breastfed at 6 mo. (n=71)			
Antibiotics <6 months	No	28	1.
	Yes	43	0.76 (0.50, 1.13)
Number of antibiotic courses†	0	28	1.
	1	20	0.73 (0.45, 1.19)
	2+	23	0.77 (0.48, 1.23)
Not exclusively breastfed at 6 mo. (n=394)			
Antibiotics <6 months	No	170	1.
	Yes	224	1.48 (1.23, 1.78)
Number of antibiotic courses†	0	170	1.
	1	115	1.53 (1.24, 1.88)
	2+	109	1.42 (1.14, 1.77)

*Incident rate ratio adjusted for covariates listed in the methods and specified in the supplemental material.

†Categorization reduced to 0, 1, and 2+ courses because of small sample size (reduced model is supported by AIC).

Table 6.5. Estimated effect of antibiotics in the first 6 months of life and exclusive breastfeeding at first antibiotic exposure on rates of diarrhea from 6 months to 3 years of age among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	No. of children	Adjusted IRR* (95% CI)
No antibiotic exposure	198	1.
Exclusively breastfed at first antibiotic exposure	163	1.28 (1.06, 1.54)
Not exclusively breastfed at first antibiotic exposure	104	1.40 (1.14, 1.74)

*Incident rate ratio adjusted for child sex, socioeconomic status, maternal education, household hygiene, household crowding, low birth weight, number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight, stunted, and wasted in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months

Table 6.6. Estimated effect of antibiotic exposure in the first 6 months of life using an alternative definition of exposure* on rates of diarrhea from 6 months to 3 years of age among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

				Incidence rate ratio (95% CI)	
		No. of children	Rate of diarrhea†	Crude	Adjusted‡
Antibiotics <6 months	No	208	10.8	1.	1.
	Yes	257	15.5	1.44 (1.21, 1.71)	1.25 (1.06, 1.48)
Number of antibiotic courses	0	208	10.8	1.	1.
	1	134	14.6	1.35 (1.11, 1.66)	1.28 (1.06, 1.54)
	2	73	16.6	1.53 (1.20, 1.96)	1.30 (1.03, 1.63)
	3+	50	16.6	1.54 (1.15, 2.06)	1.07 (0.80, 1.43)

*Children were considered exposed based on caregiver-report only if an antibiotic name was recorded
†Rate per 100 person-months
‡Adjusted for child sex, socioeconomic status, maternal education, household hygiene, household crowding, low birth weight, number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight, stunted, and wasted in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months.

Table 6.7. Estimated effect of specific antibiotics in the first 6 months of life on rates of diarrhea from 6 months to 3 years of age among 398* children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	No. of children	Adjusted IRR† (95% CI)
No antibiotics	198	1.
Amoxicillin only	68	1.46 (1.12, 1.90)
Cotrimoxazole only	79	1.32 (1.04, 1.69)
Amoxicillin and cotrimoxazole	53	1.31 (0.97, 1.76)

*67 children who were exposed an antibiotic other than amoxicillin or cotrimoxazole or to unknown antibiotics were excluded

†Incident rate ratio adjusted for child sex, socioeconomic status, maternal education, household hygiene, household crowding, low birth weight, number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight, stunted, and wasted in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months

Table 6.8. Estimated effect of antibiotic treatment of specific illnesses in the first 6 months of life on rates of diarrhea from 6 months to 3 years of age among 394* children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	No. of children	Adjusted IRR† (95% CI)
No antibiotics	198	1.
Antibiotics for diarrhea only	49	1.30 (0.94, 1.79)
Antibiotics for non-diarrheal illnesses only	147	1.41 (1.16, 1.71)

*71 children who were treated with antibiotics for both diarrhea and non-diarrheal illnesses were excluded

†Incident rate ratio adjusted for child sex, socioeconomic status, maternal education, household hygiene, household crowding, low birth weight, number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight, stunted, and wasted in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months

Table 6.9. Estimated effect of antibiotic treatment of specific illnesses in the first 6 months of life on rates of diarrhea from 6 months to 3 years of age among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

No. diarrhea episodes 0-6 months	Antibiotics <6 months	No. of children	Adjusted IRR* (95% CI)
0	No	98	1.
	Yes	67	1.31 (0.99, 1.75)
1	No	52	1.
	Yes	75	1.30 (0.96, 1.78)
2	No	34	1.
	Yes	68	1.44 (1.02, 2.03)
3+	No	14	1.
	Yes	57	1.30 (0.80, 2.10)

*Incident rate ratio adjusted for child sex, socioeconomic status, maternal education, household hygiene, household crowding, low birth weight, number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight, stunted, and wasted in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months

Table 6.10. Estimated effect of antibiotic exposures in the first 6 months of life on rates of diarrhea from 6-18 months and 18-36 months among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

Outcome		No. of children	Adjusted IRR* (95% CI)
Diarrheal rates from 6-18 months of age			
Antibiotics <6 months	No	198	1.
	Yes	267	1.30 (1.08, 1.56)
Number of antibiotic courses‡	0	198	1.
	1	135	1.32 (1.07, 1.62)
	2	74	1.28 (1.00, 1.64)
	3+	58	1.26 (0.94, 1.69)
Diarrheal rates from 18-36 months of age†			
Antibiotics <6 months	No	182	1.
	Yes	247	1.35 (1.05, 1.72)
Number of antibiotic courses	0	182	1.
	1	128	1.41 (1.07, 1.85)
	2	70	1.44 (1.04, 1.99)
	3+	49	0.90 (0.59, 1.37)

*Incident rate ratio adjusted for child sex, socioeconomic status, maternal education, household hygiene, household crowding, low birth weight, number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight, stunted, and wasted in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months

†Excludes 36 children who dropped out of the study before 18 months of age

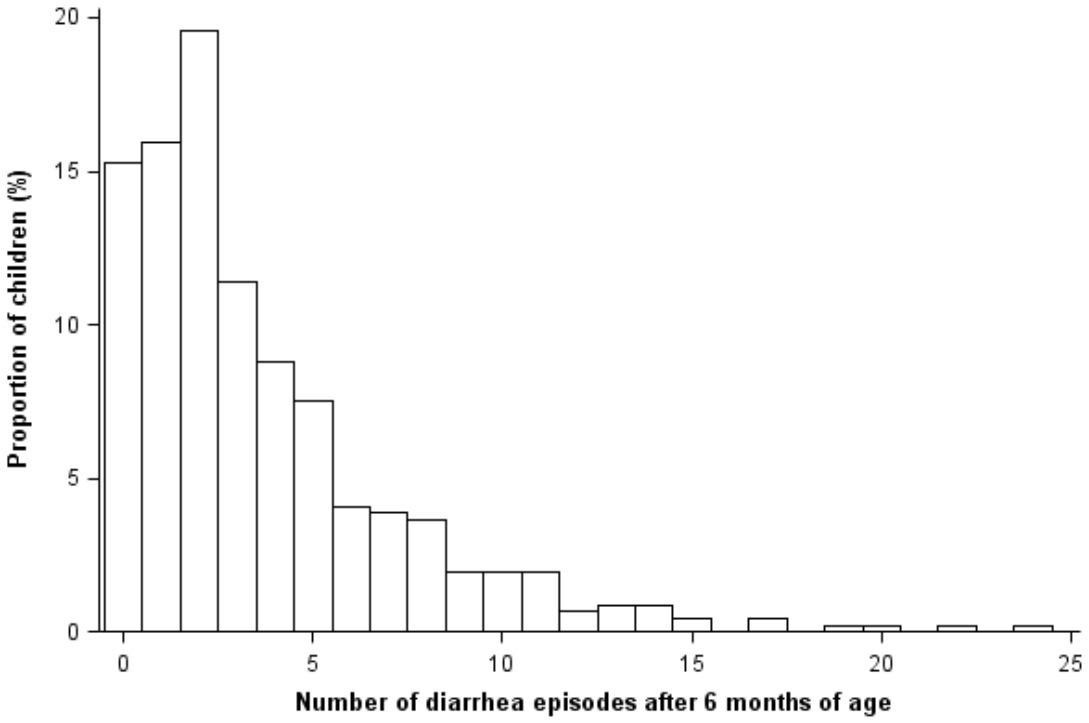

Figure 6.1. Number of diarrhea episodes after 6 months of age among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

CHAPTER VII: REDUCTION IN DIARRHEAL RATES THROUGH INTERVENTIONS THAT PREVENT UNNECESSARY ANTIBIOTIC EXPOSURE EARLY IN LIFE

Abstract

Background

Antibiotic exposure before 6 months of age has been associated with increased rates of subsequent diarrhea. We estimated the impact of realistic interventions that would prevent unnecessary antibiotic exposures early in life on childhood diarrheal rates.

Methods

In data from a prospective observational cohort study conducted in Vellore, India, we used the parametric g-formula to model diarrheal incidence rate differences contrasting the observed incidence of diarrhea to the incidence expected under hypothetical interventions. The interventions prevented unnecessary antibiotic treatments for non-bloody diarrhea, vomiting, and upper respiratory infections before 6 months of age. We also modeled targeted interventions, in which unnecessary antibiotic use was prevented only among children who had already stopped exclusive breastfeeding.

Results

More than half of all antibiotic exposures before 6 months (58.9%) were likely unnecessary. The incidence rate difference associated with removing unnecessary antibiotic use before 6 months of age was -0.28 (95% CI: -0.47, -0.11) episodes per 30 child-months. This

implies that preventing unnecessary antibiotic exposures in just 4 children would reduce the incidence of diarrhea by one from 6 months to 3 years of age.

Conclusions

Interventions to reduce unnecessary antibiotic use among young children could result in an important reduction in diarrheal rates. This work provides an example application of statistical methods which can further the aim of presenting epidemiologic findings that are relevant to public health practice.

Introduction

Antibiotic treatment of childhood illnesses is common around the world, including for uncomplicated cases of acute gastroenteritis (AGE) and upper respiratory infections (URI) [218–220]. However, antibiotic treatment is often unnecessary for these illnesses, which are usually self-limited regardless of etiology [221,222]. Further, antibiotics are not effective against the viral pathogens often responsible for these illnesses [218,223,224], and antibiotics may elicit adverse reactions or make the illness worse [191,218]. Indiscriminate antibiotic use also contributes to antimicrobial resistance and the potential for antibiotics to become ineffective for future treatment of disease [191,218,221]. Correspondingly, international organizations, including the World Health Organization, recommend against routine use of antibiotics to treat non-bloody diarrhea and URI [10,188,218].

In a recent publication, we provided evidence that antibiotic treatment of any illness early in life may increase diarrheal risk [438]. Specifically, the relative incidence rate of diarrhea from 6 months to 3 years of age was 33% higher among all children who received at least one course of antibiotics before 6 months of age compared to children who did not receive antibiotics

(adjusted IRR: 1.33, 95% confidence interval: 1.12, 1.57). There was effect modification by exclusive breastfeeding, such that children who were exclusively breastfed until at least 6 months of age did not have increased diarrheal rates associated with antibiotic exposure [438]. We hypothesize that these effects were mediated by the gastrointestinal microbiota, which is important for intestinal immune function [433,434]. Antibiotics alter the composition of the microbiota [17], and have been associated with increased intestinal inflammation, intestinal permeability, and susceptibility to infections [433,434].

In this previous work, we compared a counterfactual scenario in which all of the children were exposed to antibiotics before 6 months to a scenario in which none of the children were exposed to antibiotics before 6 months. This all-versus-none contrast is the default effect reported in most statistical analyses and is termed in the methodological literature as the average treatment effect or population average causal effect [425]. This contrast implies an intervention that would remove all antibiotic exposures before 6 months of age. However, some illnesses require antibiotic treatment, and the benefits of curing these illnesses likely outweigh any costs associated with future diarrheal risk. Therefore, the population average causal effect represents an intervention that is unrealistic and unethical.

A more plausible public health intervention would be one that prevents only *unnecessary* antibiotic use, such as antibiotic exposures for the treatment of AGE without bloody diarrhea and URI. Here, we used the parametric g-formula [420,421,424,425] to estimate intervention effects that are more relevant to public health policy in addition to the usual exposure effects [420,425,446]. Specifically, we estimated the effects of interventions that would remove only unnecessary antibiotic exposures before 6 months of age, both in the general study population and when targeted to children no longer exclusively breastfed since children who stopped

exclusive breastfeeding before 6 months of age had the greatest increase in diarrheal risk associated with antibiotics [438].

Methods

We analyzed data from a prospective observational cohort study of immune responses in cryptosporidiosis in 497 children followed from birth to 3 years of age. The study population consisted of all children born in four geographically adjacent, semi-urban slums of Vellore, Tamil Nadu, India between April 2009 and May 2010. Children were followed twice-weekly for diarrhea episodes, defined as at least three loose or watery stools in a 24-hour period [10], and antibiotic use. Other illnesses were assessed and treated at a conveniently located and free study clinic. The study population, enrollment strategy, and data collection methods have been previously described [28]. The data and analytic definitions were described in our previous analysis of the effect of early life antibiotic use on diarrheal rates in this study population [438].

Briefly, we used negative binomial regression to estimate IRRs for diarrhea from 6 months to 3 years comparing children who received any antibiotics before 6 months of age to children who did not. Because we did not detect a dose-response relationship between the number of antibiotic courses received and diarrheal rates, we used a binary classification of antibiotic exposure comparing at least one antibiotic course received to no courses received. We adjusted for demographic characteristics and measures of illness in the first 6 months as indicated in the footnote of Table 2.

To classify potentially unnecessary antibiotic use, we characterized antibiotic treatments by indicating diagnosis: AGE (further categorized into bloody diarrhea, non-bloody diarrhea, or vomiting only), URI, and other. Diagnoses for diarrhea and presence of bloody stools were

recorded in the cohort study data. Diagnoses for all other illnesses were extracted from study clinic records as documented by clinic physicians and did not correspond to specified illness definitions. We classified antibiotics for non-bloody diarrhea as "not indicated" according to clinical guidelines given our confidence in the diarrhea case definition. We considered antibiotics for URI and vomiting as "likely not indicated" to reflect the potential variability and uncertainty in clinic diagnoses. Antibiotics given for all other illnesses, including cases of bloody diarrhea, were considered necessary.

Statistical methods

We used the parametric g-formula [420–425] to estimate contrasts associated with the effect of antibiotic use on diarrheal rates. Broadly, the parametric g-formula can be understood as a parametric approach to standardization to specific covariate and exposure distributions [422]. More specifically, the procedure for fitting the g-formula was as follows: we 1) estimated beta coefficients for the observed exposure and covariates using the negative binomial model with rates of diarrhea from 6 months to 3 years as the outcome; 2) used the estimated coefficients to predict the incidence rate of diarrhea in all individuals under the index exposure and again under the referent exposure; 3) averaged the predicted outcomes across individuals in the exposure groups; and 4) compared the average outcomes to estimate the population-standardized rate difference. Confidence intervals were constructed by bootstrap of the above steps with 200 replicates [419]. We also estimated the number needed to treat (NNT) for each contrast as the reciprocal of the rate difference. In this setting, the NNT is more precisely the "number needed to intervene" by withholding unnecessary antibiotic treatment in the first 6 months of life. However, we maintain the usual terminology here for simplicity. Because the NNT is calculated

from the rate difference, it is interpreted as the NNT to see a one episode reduction in diarrhea incidence over the 30-month period from 6 months to 3 years of age. The parametric g-formula in this setting is equivalent to parametric standardization to the full population distribution of covariates [422].

We considered two interventions: (i) removing all antibiotics that were classified as not indicated before 6 months of age, and (ii) additionally removing those likely not indicated before 6 months of age. All other antibiotic exposures were not affected by the simulated interventions. Given our binary exposure classification (exposed to at least one course of antibiotics versus none), children remained exposed to antibiotics if they had any necessary antibiotic exposures. Children who received only unnecessary antibiotics moved from exposed to unexposed after the interventions. When targeted, the interventions were applied only to children who were treated after they had stopped exclusive breastfeeding.

The index and referent exposures are described for each contrast in Table 1. The referent exposures correspond to the *observed* exposures in all cases except for the population average causal effect, in which the referent is a counterfactual scenario in which all children were treated with at least one course of antibiotics. The index exposures refer to counterfactual scenarios that would occur if all antibiotic exposures were removed (in the cases of the population average causal effect and population attributable contrast) or if the interventions were to be implemented (in the cases of the generalized and targeted intervention contrasts).

In sensitivity analyses, we estimated the population average and generalized intervention contrasts in the exposed population only. These effects, commonly termed the "effect of treatment in the treated," estimate the contrasts for a target population with the same distribution of covariates as the *exposed* population instead of as the *total study* population. Correspondingly,

the parametric g-formula in this setting is equivalent to a parametric approach to standardization to the exposed population distribution of covariates [422]. The referent and index exposures are the same as those in the corresponding contrasts in the total study population. These effects are appropriate when effect measure modification is expected by covariates that differ between the exposed and unexposed groups [447–449].

In a second sensitivity analysis, we expanded our models to estimate separate coefficients for the effects of necessary and unnecessary antibiotics and included the interaction between them to account for any differences in the antibiotic effect by indicating condition.

Results

Among 465 children in the parent cohort who remained in the study for more than 6 months (93.6%), 25.4% and 12.6% of antibiotic exposures from birth to 3 years of age were given for non-bloody diarrhea (not indicated) and URI or vomiting (likely not indicated) respectively (Figure 1). More than half (n=267, 57.4%) of children were given at least one course of antibiotics in the first 6 months of life, among whom the median number of antibiotic courses received was one (mean=1.9, SD=1.14). Nearly one-third of antibiotics before 6 months (32.3%) were not indicated according to our classification, and another 26.6% were likely not indicated. Under Intervention (i), which removed antibiotics that were not indicated (32.3%), 217 children (46.7%) remained exposed to necessary antibiotics. Under Intervention (ii), which removed antibiotics that were not or likely not indicated before 6 months of age (58.9%), only 162 children (34.8%) remained exposed, resulting in more than a 20% absolute reduction in exposed children. The average length of follow-up was 2.29 years (27.24 months).

The effect estimates for each contrast are shown in Table 2. The rate difference associated with the population average causal rate difference was the largest in magnitude (incidence rate difference (IRD): -1.11 diarrhea episodes per 30 person-months, 95% CI: -1.96, -0.47) since this effect represents the most extreme contrast (all children versus none exposed to antibiotics) and does not correspond to a realistic reduction in antibiotic use. The population attributable incidence rate difference (IRD: -0.67 episodes per 30 person-months, 95% CI: -1.14, -0.27) was smaller since the exposure was unchanged among the 42.6% of children who were not exposed to antibiotics before 6 months of age in this index scenario.

We then estimated the contrasts associated with the impact of implementing the hypothetical interventions to reduce antibiotic use. The implementation of Intervention (i) in the total study population—removing antibiotic treatment for non-bloody diarrhea—would result in 0.15 fewer diarrhea episodes per child on average from 6 months to 3 years of age (IRD: -0.15 episodes per 30 person-months, 95% CI: -0.27, -0.04; Table 2). Further removing antibiotics for URI and vomiting in Intervention (ii) would result in nearly double that effect: 0.28 fewer diarrhea episodes per 30 person-months (IRD: -0.28 episodes per 30 person-months, 95% CI: -0.47, -0.11).

The effects of the interventions were smaller in magnitude than the population average causal rate difference since the interventions would remove only a proportion of (rather than all) antibiotic exposures. Comparatively, the generalized intervention rate difference for Intervention (i) was 14% of the population average causal rate difference and 25% of this effect for Intervention (ii), which removed a greater proportion of antibiotics.

The targeted intervention rate differences were smaller in magnitude than the generalized intervention rate differences because while the majority of children stopped exclusive

breastfeeding before 6 months (n=394, 84.7%), over half of antibiotic exposures occurred while the children were still exclusively breastfed (55.5%) and were therefore not removed by the targeted intervention. Intervention (ii) implemented only after children stopped exclusively breastfeeding would result in 0.17 fewer diarrhea episodes on average per child over the 30 months from 6 months to 3 years of age (IRD: -0.17 episodes per 30 person-months, 95% CI: -0.27, -0.10; Table 2).

The corresponding NNTs were very low for these effects (Table 2). Assuming the generalized intervention rate difference for Intervention (ii) was unbiased, we would need to remove unnecessary antibiotic exposures before 6 months of age for only 3.6 children to see a reduction in diarrhea incidence by one episode during the 30 months between 6 months to 3 years of age (NNT: 3.6, 95% CI: 2.1, 9.1).

Sensitivity analyses

The population average causal and generalized intervention incidence rate differences among the children exposed to antibiotics were slightly larger in magnitude than the corresponding contrasts in the full study population since average rates of diarrhea were higher among the exposed children (4.80 episodes per 30 person-months). For example, the population average causal incidence rate difference in the exposed was -1.17 episodes per 30 person-months (95% CI: -2.01, -0.47), which is slightly larger than that in the total study population, although this result was not statistically significant. The intervention effects were also larger; the effect of Interventions (i) and (ii) in the exposed were -0.26 (95% CI: -0.47, -0.08) and -0.48 (95% CI: -0.82, -0.19) episodes per 30 person-months respectively. However, similar effects in the exposed

and total study population suggest that there were not strong effect measure modifiers of the effect of antibiotics on either the difference or ratio scales.

When allowing for different effects of necessary and unnecessary antibiotics in the models, the results did not change qualitatively. The magnitudes of the estimated contrasts were very similar, though the estimates were less precise (Table 3).

Discussion

This study provides estimates of the potential impact of interventions to reduce antibiotic use among children in the first 6 months of life. These findings are more relevant to public health policy than our previously reported population average causal effect [425,450,451], which best corresponds to patient-level effects and may be more appropriate when making individual treatment decisions. The population average causal rate difference and the population attributable rate difference do not correspond to any meaningful or expected changes in diarrheal rates on a population level because some illnesses require antibiotic treatment, and it would be unethical to remove all antibiotic exposures. By estimating the impact of removing only *unnecessary* antibiotics, the generalized intervention incidence rate differences provide a more realistic expectation of the outcomes of public health interventions.

While the estimates of these contrasts are necessarily smaller in magnitude than the population average causal effect since only a portion of antibiotic exposures would be removed, our models suggest that the proposed interventions would have an important impact on child health given the high prevalence of antibiotic treatment and risk of diarrhea in this population. The low estimated NNTs (Table 2) highlight this potential impact. Because diarrhea is almost universal and recurring among these children, even a partial reduction of antibiotic exposure

could substantially reduce diarrheal rates at the population level. This effect would improve overall child development since diarrhea is a leading cause of death among children in the developing world [1] and can lead to life-long morbidity associated with stunted growth and cognitive impairment when not fatal [3].

We do not calculate NNTs for the population average causal and population attributable incidence rate differences because these effects do not correspond to a plausible intervention, and therefore the NNTs would be misleading to interpret. The intervention that implicitly corresponds to these two estimates is one in which even necessary antibiotic exposures would be removed. This would almost certainly lead to negative outcomes associated with severe illnesses being left untreated and could potentially increase risk of death. Any such intervention would be unethical, and therefore estimating its effects would be fundamentally uninformative for public health. In addition, from a data-modeling standpoint, we do not have data concerning the complex effects of withholding antibiotic treatment for necessary illnesses that would be required for estimating the impact of such an intervention.

The rate differences for the targeted interventions were smaller than those for the generalized interventions because the targeted interventions prevented antibiotic exposures only after children stopped exclusive breastfeeding. Thus, more children remained exposed under the targeted interventions due to antibiotic use during exclusive breastfeeding. These results suggest that a general intervention applied to all children before 6 months of age would be most effective.

This study was limited by the inability to definitively characterize antibiotic treatment as unnecessary. Only information concerning the indicating illness was available, and other symptoms that may have indicated antibiotic treatment were unknown. Further, clinical criteria

for diagnoses could have varied by physician at the study clinic. A subset of URI and AGE cases could have been of bacterial etiology and responded to antibiotics. In these cases, worse outcomes due to withholding of antibiotic treatment might have outweighed effects of increased diarrheal risk. On the other hand, it is also likely that some fever cases were viral and did not require antibiotics, which would make our definition of unnecessary antibiotic use conservative. Our classification is likely reasonable since diagnostic capabilities in the area where the underlying study was conducted are not sufficient to distinguish between bacterial versus viral etiologies, and thus treatment decisions are based on clinical signs alone. Presence of bloody stools during diarrhea is a common clinical indicator that justifies antibiotic treatment [10] and is used here to distinguish cases of diarrhea for which antibiotics were or were not indicated. Because international guidelines do not recommend antibiotic treatment for the majority of cases of non-bloody diarrhea, URI, and vomiting [10,188,218], classification of these treatments as not indicated was likely warranted. However, in practice, antibiotic treatment decisions should be made on a case-by-case basis and take into account both the potential benefits and harms of antibiotic treatment.

Because there were few severe illnesses and deaths in our cohort, we retained diarrhea incidence as the main outcome of interest. However, we acknowledge that the impact of the interventions on more serious diarrhea-related outcomes may also be of interest. Given the available cohort data, we were also unable to model other potential negative outcomes of antibiotic use such as risk of adverse drug reactions, healthcare costs, and development of antimicrobial resistance.

Our effects may not represent the true impact of the interventions proposed if necessary antibiotic exposures have a different causal effect on subsequent diarrheal risk than unnecessary

antibiotics. However, we have no evidence to support such a difference and validated the assumption of consistent effects in sensitivity analyses. There were no differences in the results after adding flexibility in the models to allow for differential effects between necessary and unnecessary antibiotic exposures. In addition, there was no difference in the effect of antibiotics on diarrheal rates in our previous work [438] depending on the indicating illness for antibiotic treatment.

Finally, our use of the g-formula relied on parametric modeling, which like other models, may have been misspecified. However, we expect our model to be appropriate given the model-predicted outcomes matched the observed incidence. The consistency of results in sensitivity analyses further support the assumption of no model misspecification. Because our models did not include a dose-response relationship between diarrheal rates and the number of antibiotic courses received, children who had at least one necessary antibiotic exposure remained exposed under the interventions. Our estimates are therefore likely conservative since they ignore the possibility of a benefit due to reducing, but not eliminating, all antibiotic exposures for a given child.

To understand the impact of early life antibiotics on diarrheal risk, we used the parametric g-formula as a unifying method to estimate multiple exposure and intervention contrasts. The parametric g-formula in the time-fixed setting is relatively straightforward to implement, in contrast to its application in the time-varying setting [424,425]. We suggest that the parametric g-formula is a viable alternative to regression modeling that allows simple extensions to estimate population intervention effects in addition to exposure effects [425]. The method is also useful for quantitatively comparing potential interventions, such as universal versus targeted interventions, which have been the subject of much debate [452]. Here, we show

that interventions to reduce unnecessary antibiotic use among young children could substantially reduce diarrheal rates. This work responds to recent calls for a consequentialist epidemiology [453] by providing an example application of methods which can further the aim of presenting epidemiologic findings that are relevant to public health practice and implementation science.

Table 7.1. Referent and index exposure distributions for effect contrasts.

Contrast	Referent exposure	Index exposure
Population average causal effect	The counterfactual exposure distribution had all children been treated with at least one course of antibiotics	The counterfactual exposure distribution had no children been treated with any antibiotics
Population attributable contrast	The observed exposure distribution among all children	The counterfactual exposure distribution had no children been treated with any antibiotics
Generalized intervention contrast	The observed exposure distribution among all children	The counterfactual exposure distributions after each intervention (above, i and ii) among all children
Targeted intervention contrast	The observed exposure distribution among all children	The counterfactual exposure distributions after each intervention (above, i and ii) only among children who were no longer exclusively breastfed at 6 months of age*

*Exposures for children who were exclusively breastfed until at least 6 months did not change from the observed

Table 7.2. Estimated population-level impact of antibiotic exposure before 6 months of age and of potential interventions to reduce exposure on rates of diarrhea from 6 months to 3 years among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

Contrast	Number exposed	Mean rate of diarrhea*	Incidence rate* difference (95% CI)	Number needed to treat[§] (95% CI)
Population average causal incidence rate difference				
All exposed	465	4.47	0.	
None exposed	0	3.36	-1.11 (-1.96, -0.47)	
Population attributable incidence rate difference				
Observed	267	4.04	0.	
None exposed	0	3.37	-0.67 (-1.14, -0.27)	
Generalized intervention incidence rate difference				
Observed	267	4.03	0.	
Intervention (i)[†]	217	3.88	-0.15 (-0.27, -0.04)	6.7 (3.7, 25.0)
Intervention (ii)[‡]	162	3.75	-0.28 (-0.47, -0.11)	3.6 (2.1, 9.1)
Targeted intervention incidence rate difference				
Observed	267	4.03	0.	
Intervention (i)[†] in children if no longer exclusively breastfed	237	3.91	-0.12 (-0.20, -0.07)	8.3 (5.0, 14.3)
Intervention (ii)[‡] in children if no longer exclusively breastfed	220	3.86	-0.17 (-0.27, -0.10)	5.9 (3.7, 10.0)

*Model estimated rate per 30 person-months from 6 months to 3 years of age, adjusted for exclusive breastfeeding at 6 months of age including an interaction with antibiotic exposure, child sex, socioeconomic status based on the Kuppuswamy scale [431], maternal education, household hygiene [432], household crowding, low birth weight (<2.5 kg), number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score [412] of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight (average weight-for-age z-score before 6 months of age <−2 SD from the 2006 WHO growth reference [435]), stunting (average height-for-age z-score <−2 SD), and wasting (average weight-for-height z-score <−2 SD) in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months

†Intervention (i) – removes all antibiotics for the treatment of non-bloody diarrhea (32.3% of antibiotics before 6 months of age)

‡Intervention (ii) – removes all antibiotics for the treatment of non-bloody diarrhea, upper respiratory infection, and vomiting (58.9% of antibiotics before 6 months of age)

§The number of children for whom we would need to prevent unnecessary antibiotic use in the first 6 months of life to expect a one episode reduction in diarrhea incidence over the 30-month period from 6 months to 3 years of age

CI – confidence interval by bootstrap with 200 resamples

Table 7.3. Estimated population-level impact of antibiotic exposure before 6 months of age and of potential interventions to reduce exposure on rates of diarrhea from 6 months to 3 years including separate effects for necessary and unnecessary antibiotics among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

Contrast	Number treated	Mean rate of diarrhea*	Incidence rate* difference (95% CI)	Number needed to treat[§] (95% CI)
Population average causal incidence rate difference				
All treated	465	4.46	0.	
None treated	0	3.37	-1.10 (-2.23, -0.21)	
Population attributable incidence rate difference				
Observed	267	4.04	0.	
None treated	0	3.37	-0.67 (-1.11, -0.26)	
Generalized intervention incidence rate difference				
Observed	267	4.04	0.	
Intervention (i)[†]	217	3.86	-0.18 (-0.35, 0.06)	5.6 (2.9, -)
Intervention (ii)[‡]	162	3.72	-0.32 (-0.62, 0.15)	3.1 (1.6, -)
Targeted intervention incidence rate difference				
Observed	267	4.04	0.	
Intervention (i)[†] in children if no longer exclusively breastfed	237	3.85	-0.19 (-0.43, -0.05)	5.3 (2.3, 20.0)
Intervention (ii)[‡] in children if no longer exclusively breastfed	220	3.80	-0.24 (-0.48, -0.08)	4.2 (2.1, 12.5)

*Model estimated rate per 30 person-months from 6 months to 3 years of age, including separate effects for necessary and unnecessary antibiotics and their interaction. Model adjusted for exclusive breastfeeding at 6 months of age including an interaction with antibiotic exposure, child sex, socioeconomic status based on the Kuppuswamy scale [431], maternal education, household hygiene [432], household crowding, low birth weight (<2.5 kg), number of diarrhea episodes in first 6 months, total number of days with diarrhea in first 6 months, maximum Vesikari score [412] of diarrhea episodes in first 6 months, number of severe (Vesikari ≥ 11) episodes in first 6 months, prolonged or persistent diarrhea episode in first 6 months, hospitalization for diarrhea in the first 6 months, fever during diarrhea in first 6 months, dehydration during diarrhea in first 6 months, underweight (average weight-for-age z-score under 6 months of age <−2 SD from the 2006 WHO growth reference [435]), stunting (average height-for-age z-score <−2 SD), and wasting (average weight-for-height z-score <−2 SD) in the first 6 months, any severe illness in first 6 months, number of other infections in first 6 months

†Intervention (i) – removes all antibiotics for the treatment of non-bloody diarrhea

‡Intervention (ii) – removes all antibiotics for the treatment of non-bloody diarrhea, upper respiratory infection, and vomiting

§The number of children for whom we would need to prevent unnecessary antibiotic use in the first 6 months of life to expect a one episode reduction in diarrhea incidence over the 30-month period from 6 months to 3 years of age

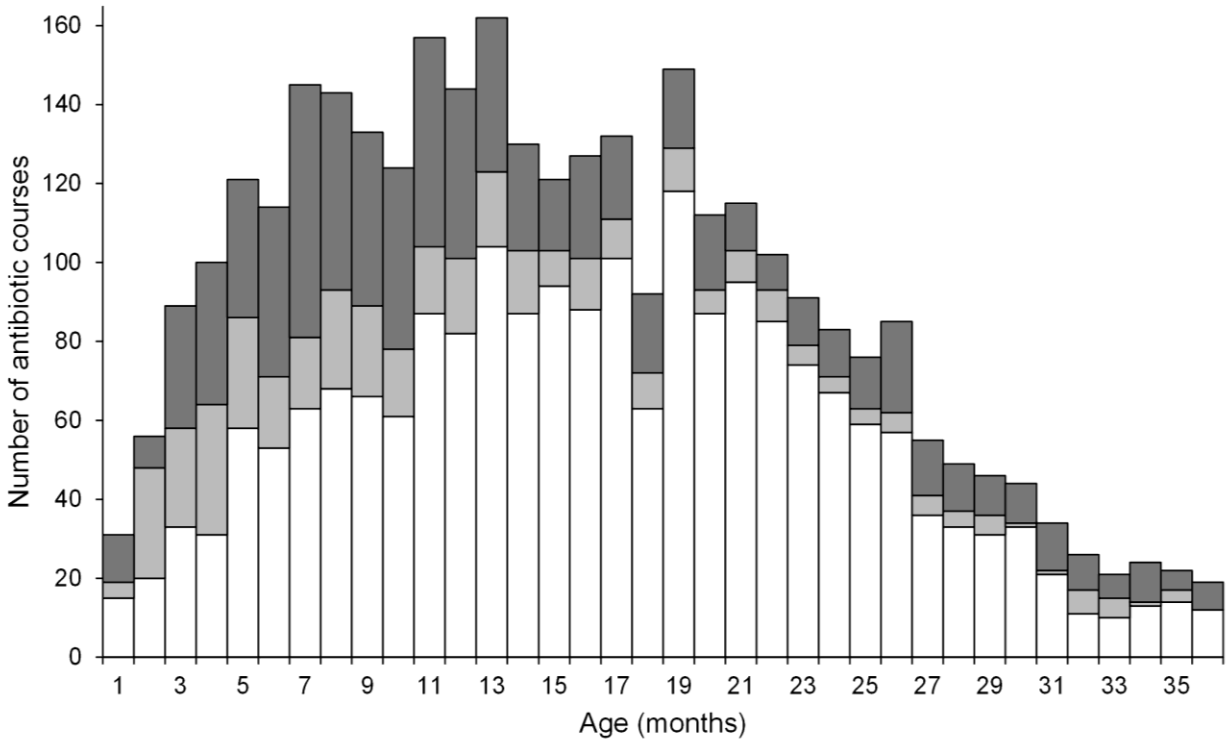

Figure 7.1. Number of antibiotic courses received by age among 465 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013. Dark gray – antibiotics given for non-bloody diarrhea (not indicated); light gray – antibiotics given for upper respiratory infections and vomiting (likely not indicated); white – antibiotics given for other illnesses.

CHAPTER VIII: NO ASSOCIATION OF EARLY LIFE ANTIBIOTIC EXPOSURE WITH GROWTH IN YOUNG CHILDREN OF VELLORE, INDIA

Abstract

Background

Early antibiotic exposure has recently been associated with increased weight gain in children in high-income countries. However, antibiotic use early in life has also been associated with increased diarrheal risk, which could contribute to poor growth outcomes. The net effect of antibiotic exposures on growth among children in low and middle-income countries is unknown.

Methods

We estimated the effects of antibiotic exposures in the first 6 months of life on short- and long-term growth. Short-term effects were measured during the first 6 months, using longitudinal general linear regression to model weight-for-age, height-for-age, and weight-for-height z-scores in monthly intervals. To estimate long-term effects, we modeled growth measurements from 6 months to 3 years of age as a function of antibiotic use in the first 6 months. We also estimated the effects of antibiotics on the monthly relative risks of underweight, stunting, and wasting in the first 6 months and to 3 years.

Results

Underweight, stunting, and wasting were common in this population: 31%, 32%, and 15% on average after 6 months of age respectively. There was no association between antibiotic

exposures before 6 months and growth during that period. From 6 months to 3 years, adjusted absolute differences in size were small (approximately -100 g and no more than -2 mm overall) and not statistically significant.

Conclusions

Antibiotic exposures early in life were not associated with increased or decreased growth. The combination of malnutrition and recurrent illness likely complicate the relationship between antibiotic exposures and growth among children in low and middle-income countries.

Introduction

In an era of concern over the growing obesity epidemic in developed countries, antibiotic exposures early in life have been recently identified as a potential contributor to excessive weight gain [24,454]. This hypothesis originated from the clear growth-promoting effects of antibiotics in livestock when given long-term and in sub-therapeutic doses [361]. While the biological mechanism is largely unknown, it is hypothesized that antibiotics affect growth through interaction with the GI microbiota [361,455], which plays an important role in supporting nutrient absorption and other metabolic functions [22,23]. Several epidemiologic studies among children in high-income countries have supported this hypothesis, finding associations between antibiotic use early in life and increased risk for obesity in later childhood [11,228,373].

In LMICs, this potential relationship is complicated by the high prevalence of malnutrition and recurrent illnesses that can cause major height and weight shortfalls [109,110]. Subclinical infections associated with living in unhygienic environments are associated with environmental enteropathy, which results in impaired function and structure of the small intestine and reduces nutrient absorption [3,161,163]. For children with severe acute

malnutrition, antibiotics are recommended to treat and prevent subclinical infections in an effort to improve recovery [456,457]. In several studies, acutely malnourished children who received antibiotics showed improved recovery rates, lower mortality, and in some cases improved weight gain compared to children receiving nutritional supplements alone [369,457].

However, as our group showed recently, antibiotics may also increase risk for diarrhea [438,458], which is associated with poor growth and can contribute to the synergism between infections and growth failure [111]. Therefore, it is unclear what the net impact of antibiotic treatment for common childhood illnesses may be among children in low-income settings.

We aimed to estimate the effect of antibiotic use before 6 months of age on short-term (within the first 6 months) and long-term (up to 3 years of age) growth in an observational birth cohort from Vellore, India. We focused on antibiotic use in the first 6 months since antibiotics have the largest impact on the developing microbiota [20,24,280] and subsequent diarrhea at this age [438], and we expect this exposure period to correspondingly have the largest effects on growth, as previously shown [11].

Methods

We analyzed data from a prospective observational cohort study of immune responses to cryptosporidiosis in 497 children from semi-urban slums of Vellore, India from 2009-2013. The study population, enrollment strategy, and data collection methods have been previously described [28]. Briefly, baseline information on demography, socioeconomic indicators, environment, and delivery characteristics were collected within 45 days of birth. Fieldworkers interviewed caregivers twice per week from birth to 3 years of age about all illnesses since the last visit, and further recorded details of diarrhea severity, hospitalization, and treatments given.

Antibiotic use was reported by caregivers at birth and for diarrhea, and antibiotic prescriptions for other illnesses were extracted from study clinic records.

Height and weight were measured each month of follow-up at the study clinic using single measurements. Weight was measured using a Salter weighing scale to the nearest 100 grams. Recumbent length was measured using a standard infantometer for the first year of life, and subsequently height was measured with a stadiometer, both to the nearest millimeter. Biologically implausible height and weight values were discarded, and we considered measurements taken within one week before or after a child's monthly birth anniversary as their weight/height at that month of age.

We used the 2006 WHO child growth standards [435] as the reference population to calculate weight-for-age (WAZ), height-for-age (HAZ), and weight-for-height (WHZ) z-scores. Children were classified as underweight (weight-for-age z-score < −2 SD from the growth reference), stunted (height-for-age z-score < −2 SD), and/or wasted (weight-for-height z- score < −2 SD). We also translated the effects on z-scores to their equivalents in absolute height and weight using the age and gender-specific SD differences in weight/height from the WHO expanded z-score tables [426,427].

Because the proportion of missing data was less than 3% for all baseline variables (see footnote in Table 8.1), we imputed the median values of variables for individuals with missing data.

Data analysis – Short-term effects

We used longitudinal general linear regression to model WAZ, HAZ, and WHZ at monthly intervals from 0 through 5 months of age. Specifically, we estimated the effects of

antibiotic exposures in a given month on WAZ, HAZ, and WHZ at the end of the following month (conceptually depicted in Figure 4.2A), and accounted for correlation between outcomes from the same child using GEE with a robust variance estimator. This model structure allowed for a one month lag between antibiotic exposure and the measurement of growth outcomes. To assess the sensitivity of results to this lag period, we repeated the analyses with outcomes both at the end of the same month as the exposure (Figure 4.2B; 0 months exposure lag) as well as at two months following the exposure month (Figure 4.2C; 2 months exposure lag).

Confounding variables for the exposure model were chosen by a causal DAG [415] to account for determinants of antibiotic use which also affect child growth, including growth status before exposure, other baseline characteristics, and illness burden. These variables were included to isolate the effects of antibiotics from the underlying conditions for which they were given. Optimal variable coding was determined by QIC, which is appropriate for GEE models [428].

The final models included child sex, socioeconomic status (based on the modified Kuppuswamy scale [431]), maternal education, household hygiene [432], household crowding, low birth weight (<2.5 kg), preterm birth (<37 weeks of gestation), and characteristics of the exposure month: growth z-score at the beginning of the month, exclusive breastfeeding, number of days with infections and severe illnesses, number of days with diarrhea, severe diarrhea episodes (Vesikari score [412] ≥ 11), prolonged or persistent diarrhea episodes (≥7 days), dehydration during diarrhea, ORS given during diarrhea, hospitalization, and days with diarrhea in the previous month. We separately stratified effects using interaction terms by month of antibiotic exposure, gender, exclusive breastfeeding in the exposure month, baseline malnutrition status (underweight, stunted, or wasted), and illness burden.

To validate our results with an alternate model that eliminates potential unmeasured child-level confounding, we used a fixed-intercept model in which the effects of antibiotic use in monthly intervals were estimated within-child (a child's exposed and unexposed months served as the index and reference exposures respectively) [429]. We used the robust variance estimator to account for correlation between observations within-child and necessarily included only the time-varying covariates [429] listed above.

Data analysis – Long-term effects

We created descriptive height and weight growth curves after 6 months of age by grouping measurements by month and averaging heights and weights across children given the same number of antibiotic courses before 6 months.

We then used longitudinal general linear regression with GEE to model all WAZ, HAZ, and WHZ after 6 months of age as a function of antibiotic use in the first 6 months. We included the corresponding growth z-score at 6 months in the models to account for differences in growth occurring prior to and during the antibiotic exposure period; this ensured estimation of long-term effects on growth rates following 6 months of age. Baseline confounding variables included all those in the short-term analysis, as well as Cesarean section birth. Indicators of illness burden were summarized over the first 6 months as: total number of days with diarrhea, infections, and severe illnesses, maximum Vesikari score [412] of diarrhea episodes, prolonged or persistent diarrhea episodes, and fever or dehydration during diarrhea, and exclusive breastfeeding at 3 months. We stratified effects by sex, number of antibiotic courses received before 6 months, and age period of growth (6 months-1 year, 1-2 years, 2-3 years). We further assessed modification of effects by exclusive breastfeeding, illness burden, and malnutrition status at 6 months.

We estimated the effects of antibiotics on the relative risks of underweight, stunting, and wasting in both the short and long-term with the same exposure groups and covariates as the linear regression models. We used longitudinal Poisson regression with the robust variance estimator as an approximation of log-binomial regression [430] since the log-binomial models did not converge.

We validated results by repeating analyses with a more specific, but less sensitive, definition of antibiotic exposure which required the caregiver to list a confirmed antibiotic name for diarrhea instead of only replying 'yes' when asked if antibiotics were given.

Results

The birth cohort consisted of 497 children, 456 (91.8%) of whom remained in the study and were measured at least once after 6 months of age. In the remaining study period, 46 (9.3%) more children were lost to follow-up. Nine drop-outs were due to death. The majority of participants were from families of low socioeconomic status with poor household hygiene and crowding in the home (Table 8.1).

More than half of children (n=262, 57.5%) were exposed to antibiotics by 6 months of age, and 137 (28.1%) had received more than one course (Figure 8.1). Antibiotic use was highest from 3-5 months of age, with an average monthly exposure prevalence of 20.3%.

Growth failure early in life was common (Figure 8.2). By 6 months, on average 30.6% of children were underweight (maximum prevalence 40.7% at 27 months) and 31.8% of children were stunted (maximum prevalence 34.7% at 32 months). Prevalence of wasting (WHZ < -2 SD) was lower, at 15.0% overall.

Short-term antibiotic effects

Averaged across months from birth through 5 months, there was no crude difference in WAZ or WHZ associated with antibiotic exposure in a given month (WAZ difference: 0.01, 95% CI: -0.05, 0.06; WHZ difference: 0.05, 95% CI: -0.04, 0.14). These effects were uniform by sex. Conversely, antibiotic use was crudely associated with slightly lower HAZ (HAZ difference: -0.12, 95% CI: -0.19, -0.06); this effect was more pronounced among girls (HAZ difference: -0.17, 95% CI: -0.26, -0.07) than boys (HAZ difference: -0.09, 95% CI: -0.17, 0.00).

After multivariable adjustment, the absence of effects on WAZ and WHZ remained. The effect on HAZ was no longer significant and moved toward the null, entirely for boys and reduced by more than half among girls (Table 8.2). The adjusted weight and height differences among boys translated to a difference of -1 g and -0.1 mm respectively. Among girls, the effects corresponded to differences of -32 g and -1.2 mm. Effects were largest for exposures in the first month of life, but were imprecise due to few exposed children (n=30). There was no statistically significant effect modification by month (*p* for heterogeneity=0.7; Table 8.3), malnutrition status of the child (underweight, stunted, or wasted), exclusive breastfeeding, diarrhea burden, or other infections and severe illness burden (results not shown).

There was also no difference in the relative risks of underweight or wasting among children who received antibiotics compared to those who did not (Table 8.4). However, girls who received antibiotics in a given month had a higher risk of being stunted in the next month (RR: 1.27, 95% CI: 1.04, 1.56). There was no effect on stunting among boys.

Long-term antibiotic effects

For the analysis of the effect of antibiotics before 6 months of age on growth from 6 months to 3 years, a total of 12,694 growth measurements were available among 456 children remaining in the study at 6 months (mean of 27.8 measurements/child). The majority of children (n=388, 85.1%) had at least 29 growth measurements before 3 years of age.

Crude average growth curves after 6 months of age stratified by gender and early life antibiotic exposure are shown in Figure 8.3. Children receiving no or one course of antibiotics had similar growth trajectories, while those receiving 2 or more courses of antibiotics weighed less and were shorter at all ages. Correspondingly, there was a crude negative association of antibiotic use in the first 6 months on WAZ (difference: -0.18, 95% CI: -0.35, -0.02) and HAZ (difference: -0.20, 95% CI: -0.38, -0.02) from 6 months to 3 years of age.

Adjusted effects were smaller in magnitude and no longer statistically significant, but still negative, such that antibiotic use before 6 months of age was associated with lower WAZ, HAZ, and WHZ after 6 months (Table 8.5). The associations were largest after 1 year of age (p for heterogeneity < 0.0001). There was no evidence for a difference in effect by gender or number of antibiotic courses received (p for heterogeneity >0.4). All effects were minimal when translated to weight and height differences. The largest differences in weight (occurring at 2-3 years of age) corresponded to approximately -150 g. The largest differences in height were -3.1 mm from 2-3 years, and the difference was -1.5 mm overall. There was no significant effect modification by burden of illnesses, hospitalization, baseline malnutrition status, or exclusive breastfeeding (results not shown).

There was an increase in the relative risk of underweight after 6 months of age among children who received antibiotics in the first 6 months compared to children who did not receive

antibiotics (RR: 1.33, 95% CI: 1.07, 1.64; Table 8.5). The risk of wasting was also elevated, but the estimates were not statistically significant. There were no effects on long-term stunting.

Sensitivity analyses

The effects of antibiotics in a given month were not sensitive to the time period between the antibiotic exposure and outcome (Figure 4.2B and 4.2C; Table 8.6). Results from the fixed-intercept model, which eliminated potential unmeasured child-level confounding, were qualitatively and quantitatively similar to results from the general linear models (Table 8.7). Using an alternative definition of exposure which required the caregiver to list a confirmed antibiotic name, there was no change in short or long-term effects (results not shown).

Discussion

Our study provides the first evidence from a prospective observational cohort study concerning the impact of early life antibiotic exposures on growth among LMIC children. Unlike other investigations of the relationship between antibiotics and growth, we did not find evidence that antibiotic exposures early in life were associated with growth promotion. Antibiotic exposures before 6 months of age did not have any short-term associations with growth, and were associated only with small, but not statistically or clinically significant, differences in height and weight from 6 months to 3 years. These differences were near the limits of detection of the measurement instruments (approximately 100 g and 1-2 mm overall). We suggest these small negative effects on growth may be due to residual confounding or chance given the large number of comparisons made.

There are several potential explanations for the lack of a growth-promoting effect. Most of the previous studies showing increased weight gain or risk of obesity associated with antibiotics [11,228,373,389,390] were conducted in high-income countries with Western diets. Animal studies have shown that the growth-promoting phenotype associated with an altered microbiota is amplified when the animals are fed a high-fat diet [24,363,459]. Our study population from semi-urban slums did not have access to a high-fat diet after weaning such that an interaction between antibiotics and increased caloric intake was unlikely. Also, no children in this study were diagnosed with acute severe malnutrition, for which antibiotics have shown to improve recovery and/or growth [369,375]. Our study population was community-based, and few would have met the inclusion criteria (e.g. preterm, very low birth weight, with severe illness) for the trials demonstrating improved growth associated with antibiotics [363,371,375,460].

In LMICs, previous studies of the effects of antibiotics on growth have been inconclusive. A recent meta-analysis of 10 trials of antibiotics conducted over a 60 year period concluded that antibiotics improved growth, though the summary effect sizes were likely not clinically important (less than 1 mm/month difference in height and 24 g/month in weight) [375]. An international cross-sectional study also reported that overall adjusted BMI at age 5-8 was higher in children exposed to antibiotics in infancy. However, the effects varied across sites and, critically, a lower in BMI associated with antibiotics was found in all countries classified as non-affluent except Thailand [376].

The small association of antibiotics with lower WAZ and HAZ in the long-term may be due to increased diarrheal rates following antibiotic exposure [438], which may be associated with poor growth. However, diarrhea likely had minimal impact on growth in our study

population due to high use of ORS during diarrhea (88%), counselling to continue breastfeeding, and good access to healthcare. Therefore, appropriate treatment may have mitigated any effects increased diarrhea burden would have had on growth. It is also possible that the two competing pathways: antibiotics as growth-promoters and antibiotics as causing future illness and harming growth, may both have been occurring, resulting in a null net effect on growth.

Because the study was observational in design, we cannot exclude the possibility of uncontrolled confounding. Even after multivariable adjustment, we may not have been able to completely capture aspects of child illness needed to separate the effects of antibiotics from their indicating illnesses. However, a randomized clinical trial would be unethical since treatment of some illnesses with antibiotics is necessary, and our study provides results in a community-based setting that may be more generalizable [461] to communities in LMICs.

The study was also limited by potential misclassification of antibiotic exposures due to recall errors among caregivers and missed antibiotic prescriptions received outside of the study clinic. However, because the clinic was located within the residential area of study subjects and provided free care and medicines, we expect almost all prescriptions to have originated in the study clinic.

The combination of malnutrition and recurrent illness complicate the relationship between antibiotic exposures and growth among children in LMICs. Our study among children in south India did not replicate previous associations between early life antibiotic use and increased growth demonstrated among children in high-income countries and when given in combination with nutritional rehabilitation for more severely malnourished children. Conversely, antibiotic exposure in the first 6 months of life was not associated with differences in growth both during the first 6 months and up to 3 years of age.

Table 8.1. Demographic characteristics of 497 children in a birth cohort in Vellore, Tamil Nadu, India 2009-2013.

	No. children (%)
Household characteristics	
Socioeconomic status*	
Low	328 (66.0)
Medium	160 (32.2)
High	9 (1.8)
Maternal education	
No formal education	184 (37.0)
Primary/middle school	167 (33.6)
Higher secondary school	129 (26.0)
College/polytechnic/professional school	17 (3.4)
Poor household hygiene†‡	256 (53.5)
Crowding	
High (>4 people/room)	164 (33.0)
Medium (3.1-4 people/room)	190 (38.2)
Low (≤ 3 people/room)	143 (28.8)
Child characteristics	
Sex of child	
Male	263 (52.9)
Female	234 (47.1)
Cesarean section	90 (18.1)
Low birth weight‡	84 (17.1)
Preterm birth	50 (10.3)
Antibiotics at birth‡	13 (2.7)
Age (months) at stopping exclusive breastfeeding (mean, SD)	3.9 (2.10)
Age (months) at stopping all breastfeeding (mean, SD)	15.9 (8.72)

*Socioeconomic status categories defined from the modified Kuppuswamy scale based on educational and occupational level of the family, house ownership, total number of rooms in the house, and household possessions [431]

†Poor household hygiene was based on a score of less than 12 on a scale developed from an assessment of water, food, and personal hygiene [432]

‡2 missing observations for hygiene; 7 missing observations for low birth weight; 12 missing observations for preterm birth; 15 missing observations for antibiotics at birth; SD – standard deviation

Table 8.2. Estimated adjusted effects of antibiotic treatment in one month on growth at the end of the following month in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013.

Antibiotics in exposure month		No. (%)	WAZ in next month β* (95% CI)	HAZ in next month β* (95% CI)	WHZ in next month β* (95% CI)
No			0.	0.	0.
Yes	Boys	155 (58.9)	-0.00 (-0.07, 0.08)	-0.01 (-0.09, 0.10)	-0.00 (-0.13, 0.13)
	Girls	121 (51.7)	-0.05 (-0.13, 0.04)	-0.06 (-0.16, 0.03)	0.05 (-0.09, 0.19)
	Overall	276 (55.5)	-0.02 (-0.08, 0.04)	-0.03 (-0.10, 0.04)	0.02 (-0.07, 0.12)

*Absolute difference in z-score adjusted for child sex, previous growth z-score, socioeconomic status [431], maternal education, household hygiene [432], household crowding, low birth weight, preterm birth, exclusive breastfeeding, infections and severe illnesses, and indicators of diarrhea severity
WAZ – weight-for-age z-score; HAZ – height-for-age z-score; WHZ – weight-for-height z-score; CI – confidence interval

Table 8.3. Stratified by month, estimated adjusted effects of antibiotic treatment in one month on growth at the end of the following month in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013.

Age at exposure	No. (%)	WAZ in next month β* (95% CI)	HAZ in next month β* (95% CI)	WHZ in next month β* (95% CI)
None		0.	0.	0.
0 months	30 (6.0)	-0.28 (-0.61, 0.05)	0.05 (-0.32, 0.42)	-0.13 (-0.62, 0.35)
1 month	48 (9.7)	-0.05 (-0.26, 0.16)	-0.10 (-0.32, 0.12)	0.11 (-0.29, 0.51)
2 months	81 (16.6)	0.03 (-0.10, 0.16)	-0.06 (-0.22, 0.10)	0.17 (-0.06, 0.40)
3 months	89 (18.5)	-0.01 (-0.11, 0.09)	-0.04 (-0.16, 0.09)	-0.00 (-0.17, 0.16)
4 months	104 (21.8)	0.01 (-0.08, 0.10)	-0.06 (-0.17, 0.05)	0.01 (-0.17, 0.19)
5 months	97 (20.7)	-0.02 (-0.14, 0.09)	0.01 (-0.12, 0.15)	-0.06 (-0.25, 0.13)

*Absolute change in z-score adjusted for child sex, previous growth z-score, socioeconomic status [431], maternal education, household hygiene [432], household crowding, low birth weight, preterm birth, exclusive breastfeeding, infections and severe illnesses, indicators of diarrhea severity, and antibiotic exposure history

WAZ – weight-for-age z-score; HAZ – height-for-age z-score; WHZ – weight-for-height z-score; CI – confidence interval

Table 8.4. Estimated adjusted effects of antibiotic treatment in one month on underweight, stunting, and wasting at the end of the following month in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013.

Antibiotics in exposure month		Underweight* RR** (95% CI)	Stunting† RR** (95% CI)	Wasting§ RR** (95% CI)
No		1.	1.	1.
Yes	Boys	0.93 (0.78, 1.11)	0.94 (0.77, 1.14)	0.94 (0.73, 1.20)
	Girls	1.07 (0.88, 1.31)	1.27 (1.04, 1.56)	0.98 (0.72, 1.33)
	Overall	0.98 (0.86, 1.13)	1.07 (0.92, 1.23)	0.96 (0.78, 1.18)

*Weight-for-age z-score < -2 SD

†Height-for-age z-score < -2 SD

§Weight-for-height z-score < -2 SD

**Relative risk adjusted for child sex, previous growth z-score, socioeconomic status [431], maternal education, household hygiene [432], household crowding, low birth weight, preterm birth, exclusive breastfeeding, infections and severe illnesses, indicators of diarrhea severity, and antibiotic exposure history

WAZ – weight-for-age z-score; HAZ – height-for-age z-score; WHZ – weight-for-height z-score; CI – confidence interval

Table 8.5. Estimated adjusted effects of antibiotic treatment under 6 months of age on growth from 6 months to 3 years in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013.

Antibiotics	WAZ	Underweight	HAZ	Stunted	WHZ	Wasted
< 6 mo.	β* (95% CI)	RR† (95% CI)	β* (95% CI)	RR† (95% CI)	β* (95% CI)	RR† (95% CI)
No (n=194)	0.	1.	0.	1.	0.	1.
Yes (n=262)	-0.08 (-0.19, 0.02)	1.33 (1.07, 1.64)	-0.05 (-0.17, 0.06)	1.07 (0.88, 1.31)	-0.10 (-0.23, 0.02)	1.25 (0.94, 1.67)
By gender						
Males	-0.08 (-0.23, 0.06)	1.39 (1.03, 1.87)	-0.02 (-0.18, 0.14)	1.03 (0.79, 1.35)	-0.15 (-0.32, 0.02)	1.37 (0.94, 2.01)
Females	-0.08 (-0.23, 0.06)	1.26 (0.96, 1.66)	-0.09 (-0.24, 0.06)	1.13 (0.87, 1.47)	-0.06 (-0.22, 0.10)	1.13 (0.76, 1.69)
By courses						
1 course	-0.09 (-0.21, 0.03)	1.48 (1.16, 1.88)	-0.03 (-0.16, 0.10)	1.08 (0.85, 1.38)	-0.11 (-0.26, 0.03)	1.35 (0.98, 1.87)
2+ courses	-0.08 (-0.21, 0.05)	1.22 (0.95, 1.55)	-0.08 (-0.22, 0.06)	1.07 (0.86, 1.34)	-0.10 (-0.24, 0.05)	1.13 (0.80, 1.60)
By age						
6 mo.-1 yr.	0.01 (-0.07, 0.08)	1.23 (0.96, 1.58)	-0.01 (-0.10, 0.08)	0.96 (0.76, 1.22)	0.01 (-0.10, 0.11)	0.94 (0.68, 1.30)
1-2 yr.	-0.12 (-0.24, 0.01)	1.46 (1.15, 1.87)	-0.06 (-0.18, 0.07)	1.16 (0.90, 1.49)	-0.15 (-0.30, -0.00)	1.36 (0.97, 1.89)
2-3 yr.	-0.11 (-0.25, 0.03)	1.25 (0.98, 1.60)	-0.09 (-0.25, 0.07)	1.07 (0.85, 1.35)	-0.12 (-0.27, 0.03)	1.37 (0.91, 2.06)

*Absolute change in z-score adjusted for child sex, previous growth z-score, socioeconomic status [431], maternal education, household hygiene [432], household crowding, low birth weight, preterm birth, Cesarean section birth, exclusive breastfeeding, infections and severe illnesses, and indicators of diarrhea severity

†Relative risk adjusted for the same covariates as above

WAZ – weight-for-age z-score; HAZ – height-for-age z-score; WHZ – weight-for-height z-score; CI – confidence interval

Table 8.6. Estimated adjusted effects of antibiotic treatment in one month on growth at the end of the month and at the end of the second following month in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013.

Antibiotics in exposure month		WAZ β* (95% CI)	HAZ β* (95% CI)	WHZ β* (95% CI)
Outcome: at end of exposure month				
No		0.	0.	0.
Yes	Boys	-0.03 (-0.10, 0.04)	-0.01 (-0.10, 0.09)	-0.03 (-0.16, 0.09)
	Girls	-0.01 (-0.09, 0.07)	-0.11 (-0.20, -0.03)	0.17 (0.04, 0.31)
	Overall	-0.02 (-0.08, 0.03)	-0.05 (-0.12, 0.01)	0.05 (-0.04, 0.16)
Outcome: at end of second following month				
No		0.	0.	0.
Yes	Boys	0.05 (-0.02, 0.12)	0.03 (-0.05, 0.12)	0.01 (-0.11, 0.12)
	Girls	-0.03 (-0.10, 0.04)	-0.05 (-0.14, 0.04)	0.01 (-0.12, 0.14)
	Overall	0.01 (-0.04, 0.07)	-0.00 (-0.07, 0.06)	0.01 (-0.08, 0.10)

*Absolute change in z-score adjusted for child sex, previous growth z-score, socioeconomic status [431], maternal education, household hygiene [432], household crowding, low birth weight, preterm birth, exclusive breastfeeding, infections and severe illnesses, indicators of diarrhea severity, and antibiotic exposure history

WAZ – weight-for-age z-score; HAZ – height-for-age z-score; WHZ – weight-for-height z-score; CI – confidence interval

Table 8.7. Estimated adjusted effects of antibiotic treatment in one month on growth at the end of the following month using the fixed-intercept model in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013.

Age at antibiotic exposure	WAZ in next month β* (95% CI)	HAZ in next month β* (95% CI)	WHZ in next month β* (95% CI)
No antibiotics	0.	0.	0.
0-5 months	0.02 (-0.04, 0.08)	-0.02 (-0.09, 0.05)	0.03 (-0.07, 0.12)
Boys	0.05 (-0.02, 0.12)	0.02 (-0.07, 0.11)	0.00 (-0.13, 0.13)
Girls	-0.01 (-0.10, 0.08)	-0.07 (-0.16, 0.02)	0.06 (-0.08, 0.20)
0-2 months	0.02 (-0.08, 0.12)	-0.02 (-0.15, 0.11)	0.09 (-0.09, 0.27)
3-5 months	0.01 (-0.05, 0.08)	-0.01 (-0.09, 0.07)	-0.01 (-0.12, 0.10)

*Absolute change in z-score adjusted for child sex, previous growth z-score, socioeconomic status [431], maternal education, household hygiene [432], household crowding, low birth weight, preterm birth, exclusive breastfeeding, infections and severe illnesses, indicators of diarrhea severity, and antibiotic exposure history

WAZ – weight-for-age z-score; HAZ – height-for-age z-score; WHZ – weight-for-height z-score; CI – confidence interval

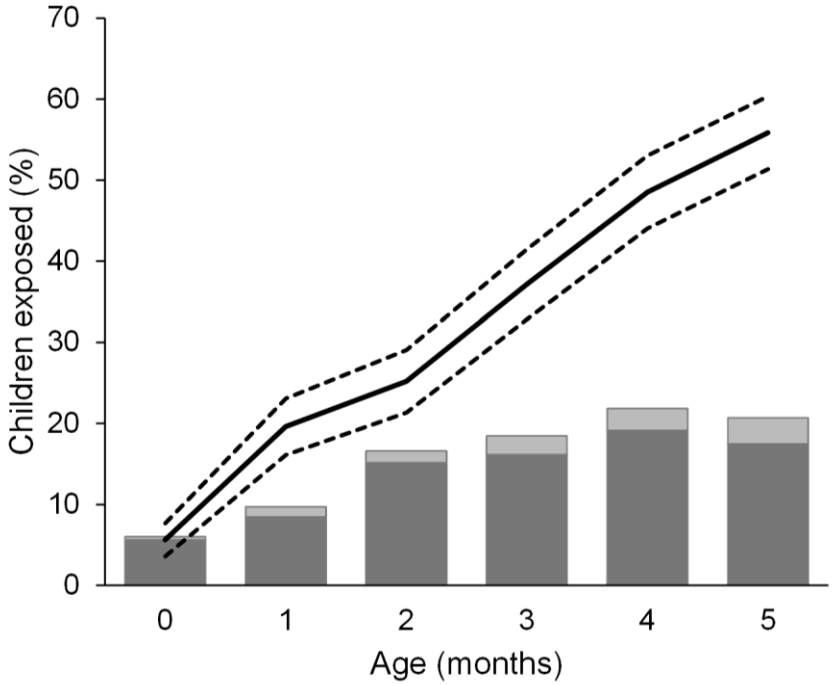

Figure 8.1. Antibiotic exposures before 6 months of age in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013. Dark gray bars – children exposed to one course of antibiotics in a given month; light gray – children exposed to more than one course of antibiotics in a given month; Black line (dotted lines) – cumulative proportion of children exposed to at least one course of antibiotics (95% confidence interval).

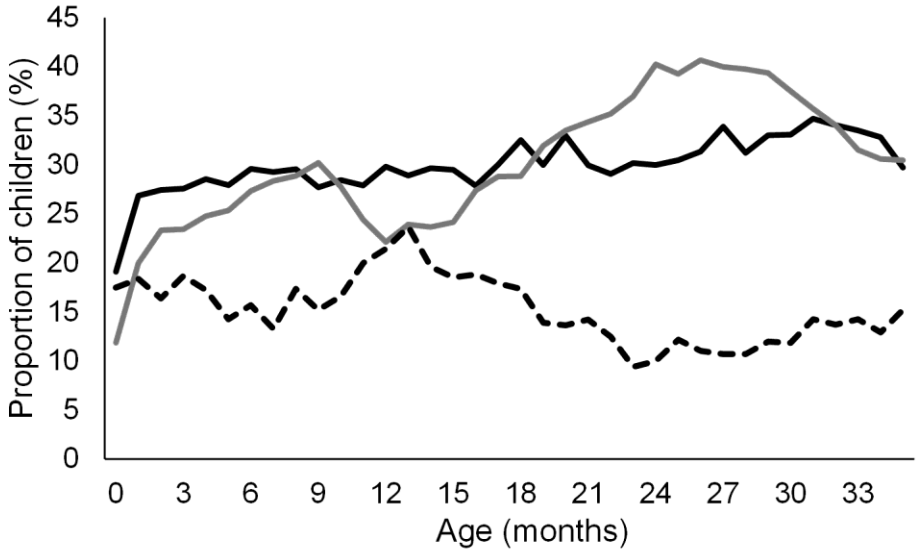

Figure 8.2. Prevalence of underweight (black line), stunting (gray line), and wasting (black dotted line) from 0-3 years of age in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013.

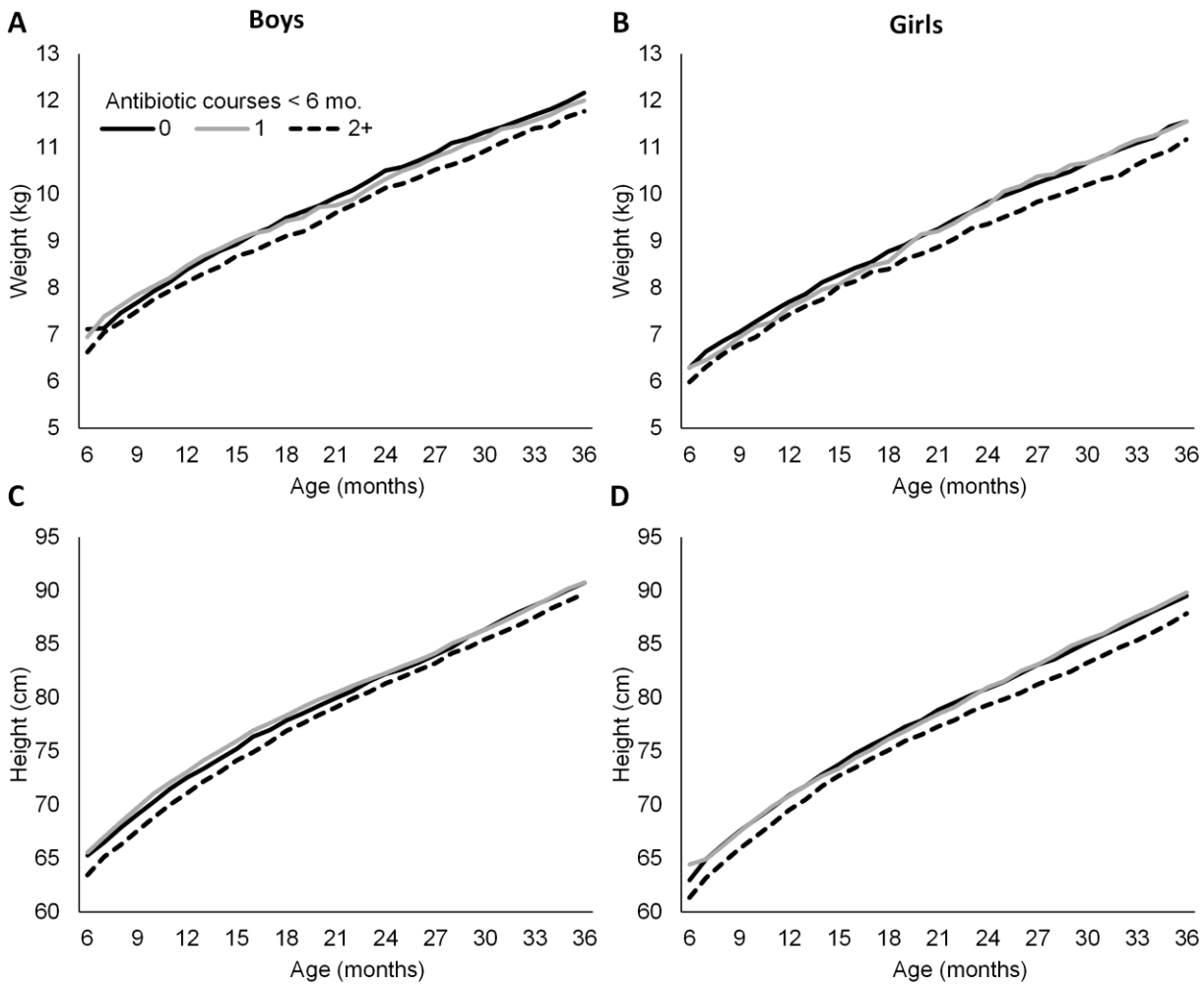

Figure 8.3. Crude average height and weight growth curves by antibiotic exposure in the first 6 months of life in a birth cohort of 497 children in Vellore, Tamil Nadu, India 2009-2013. **A** – weight among boys; **B** – weight among girls; **C** – height among boys; **D** – height among girls. Black line – no antibiotic courses; grey line – 1 antibiotic course; black dotted line – 2+ antibiotic courses.

CHAPTER IX: DISCUSSION

Antibiotics play an indispensable role in our defense against microbes and have quickly become a common exposure in early childhood. However, the unintended consequences of antibiotic treatment have not been fully realized. In addition to concerns over antibiotic resistance, the growing evidence of a disruptive effect of antibiotics on the gastrointestinal microbiota has demonstrated the potential for collateral damage to the developing immune system and metabolic pathways. These changes could further affect susceptibility to infections and growth among children. Given the high morbidity associated with childhood diarrhea and ease of access to antibiotics in India, we aimed to assess the effects of antibiotics on diarrheal risk and growth outcomes among young children in Vellore.

First, does antibiotic treatment increase risk for future diarrhea? We initially focused on antibiotic treatment of diarrhea specifically and its effects on the incidence of subsequent diarrhea. Because antibiotic treatment for diarrhea represented only a proportion of all antibiotic exposures, we then expanded our exposure definition to include any antibiotic exposures under 6 months of age. We focused on exposures in the first 6 months of life since this is the period during which the microbiota is developing and microbial communities are less able to recover from perturbations. We then estimated the impact of plausible interventions that would remove unnecessary antibiotic exposures, since antibiotic treatment of some illnesses is necessary and preventing all antibiotic exposures would be unethical. These results indicated what gains in diarrhea morbidity could be expected due to programs that enforce rational antibiotic use.

Second, does antibiotic treatment affect growth among children? This question was complicated by conflicting hypotheses that antibiotics might both promote and/or impede growth. The former is based on evidence from antibiotic-associated growth promotion in livestock and the associations of antibiotics with obesity among children in high-income countries. The latter is based on an indirect mechanism in which antibiotics may cause more diarrhea, which is then associated with poor growth. Without being able to effectively tease apart these pathways, we assessed the impact of antibiotic exposure on growth overall, in both the short and the long-term. We again focused on antibiotic exposure in the first 6 months of life given the importance of the microbiota and the increased impact of disturbances at this time. Short-term effects were estimated within monthly intervals of exposure with growth outcomes in the next month. We then assessed longer-term effects of early life antibiotic exposure by comparing growth from 6 months to 3 years among children exposed and unexposed to antibiotics under 6 months. These analyses provided the first evidence from an observational LMIC setting of the effects of antibiotics given for common childhood illnesses on growth.

Summary of findings

Diarrhea was a common and recurrent illness among children in the birth cohorts. Incidence of diarrhea was highest around 6 months of age, with an incidence of over 30 episodes per 100 person-months among children between 5 and 7 months of age. Diarrheal rates then decreased by more than half from 6 months to 3 years; still, children had more than 3 episodes on average during this period.

Antibiotics were frequently given to treat diarrhea. Nearly 30% of episodes were treated with antibiotics, and more than half of children reported at least one antibiotic course for

diarrhea. Antibiotic treatment of diarrhea was associated with older age at the time of the episode and increased episode severity and duration. The most common antibiotic given for diarrhea was cotrimoxazole, accounting for more than half of all antibiotics given. Cefixime accounted for another third, while all other antibiotics were reported for less than 5% of diarrhea cases.

More generally, antibiotic exposure due to treatment of any illness was almost universal. More than half of children were given at least one course of antibiotics in the first 6 months of life. By 1 year of age, more than 85% of children had received at least one course. This proportion increased to 94% by 3 years of age, with more than half receiving 6 or more courses. Antibiotic prescriptions were most frequently associated with diagnoses for respiratory infections, acute gastroenteritis, and acute otitis media. While only approximately 6% of upper respiratory infections were treated with antibiotics at the study clinic, the high burden of this illness resulted in many cases being treated with antibiotics. Amoxicillin was given in two-thirds of non-diarrheal diagnoses at the study clinic, with azithromycin, co-trimoxazole, cephalexin, and amoxicillin/clavulanic acid making up the majority of other prescriptions.

In the US and UK, the proportion of children treated with antibiotics under 6 months of age based on caregiver-report is reported to be lower, at approximately one third of children [11,12]. In Denmark, approximately 40% of children receive antibiotics in the first year of life as determined by antibiotic sales data [387]. Higher rates of antibiotic use in our study population may be due to higher rates of infection, better capture of antibiotic prescriptions in clinic records, and greater availability of antibiotics without prescriptions. Also, healthcare providers may prescribe antibiotics at higher rates because they perceive greater pathogen burden or lower rates of retention in care in slum areas. Reductions in antibiotic usage rates in India may be achieved by intervening on these factors. Specifically, the lack of enforcement of antibiotic purchasing

restrictions in India indicate a key potential point of intervention for preventing unnecessary antibiotic use.

Aim 1

The results from Aim 1A provide the first evidence that antibiotic treatment of diarrhea may shorten the time between episodes, especially among younger infants. Children who received antibiotics for their first diarrhea episode had their second episode on average two months earlier than children who did not receive antibiotics. The effect of antibiotics on time to next diarrhea was largest among children who were treated with antibiotics for diarrhea under 6 months of age and among children who were treated with cefixime (in contrast to cotrimoxazole). These differences aligned with expectations since the microbiota is underdeveloped and more susceptible to disturbances during early infancy [20,280]. In addition, cefixime is more effective against Gram-negatives common in the gut [439], and AAD is more commonly reported for cefixime compared to cotrimoxazole [439,440].

These conclusions were further strengthened by evidence from Aim 1B of an increase in diarrheal rates from 6 months to 3 years of age associated with any antibiotic treatment under 6 months of age, regardless of indicating illness. Antibiotic exposure during this early period of life—at the same time as the microbiota is developing—appears to have consistent negative effects on diarrheal risk. Further, we found that exclusive breastfeeding during antibiotic exposure may be protective against the effects of antibiotics since children who were exclusively breastfed for at least the first 6 months did not have increased diarrheal rates associated with antibiotic exposure.

We hypothesize that the effect of antibiotic treatment on diarrheal risk may be mediated by a prolonged effect of the antibiotics on microbiota composition [17] or through collateral effects on intestinal structure and function relating to inflammation, permeability, and intestinal immunity [247,357]. The strong effect modification by exclusive breastfeeding may also be explained by interactions with the microbiota since bacteria present in breast milk, such as *Lactobacillus*, have been shown to reduce risk of gastrointestinal infections [441,445].

To translate these results into those that would be relevant for public health practice and policy, we estimated the impact of realistic interventions (Aim 1C) that would prevent only unnecessary antibiotic use (for non-bloody diarrhea, upper respiratory infections, and other episodes of acute gastroenteritis). The interventions removed more than half of all antibiotic treatments in the first 6 months of life. While the effects on diarrheal rates were necessarily smaller than the average treatment effects presented in Aim 1B since only a portion of antibiotic exposures were removed, the interventions resulted in substantial decreases in diarrhea burden, with numbers needed to treat between 4 and 8 based on the effect contrast. When targeted to children who were no longer exclusively breastfed, the effects were slightly smaller, suggesting general interventions may be most effective. Because it would be unethical to remove all antibiotic exposures, these results provided estimates of a real population impact that could be achieved by rational antibiotic use interventions.

Aim 2

Study children fell consistently below the normal growth curve, and growth failure was common early in life. The prevalences of underweight and stunting rose quickly in the first 6

months of life from below 15% to almost 30%. After 6 months, more than 30% of children were underweight and/or stunted on average. Prevalence of wasting was lower, at 15% overall.

There were no effects of antibiotics on growth in the short-term. In primary analyses, children who received antibiotics in monthly intervals under 6 months of age did not demonstrate differences in growth compared to unexposed children. This lack of effect remained in all secondary analyses conducted within subgroups, with alternative exposure definitions, and using different models and dichotomous outcomes. In the long-term, children who received no or one course of antibiotics had similar growth trajectories, while those receiving 2 or more courses of antibiotics weighed slightly less and were shorter at all ages. However, adjusted effects were close to the null and not statistically significant. Notably, all z-score differences translated to very small equivalents in absolute weight and height (approximately 100 g and 1-2 mm), which are within the error margin of the anthropometric instruments and likely not clinically important. Because there was an increase in relative risk of underweight associated with antibiotics in the long-term analyses, it may be useful to focus future investigations of antibiotics on weight effects. However, statistical significance of effects in this case may be due to chance given the large number of comparisons made.

The lack of a growth-promoting effect of antibiotics in our study population may be explained by differences from previous studies in methodology, child diet, and indicating illnesses. Most studies demonstrating increased weight gain associated with antibiotics were conducted among those with a Western high-fat diet [11,228,373,389,390]. The lack of an effect in our population, which did not have access to a high-fat diet, suggests that the growth promoting effect of antibiotics may occur only when increased energy and nutrient intakes are possible. Further, antibiotic treatment in our study was largely given for common childhood

illnesses, and the children were not diagnosed with acute severe malnutrition for which antibiotics have shown to improve growth, especially when given in combination with nutritional supplementation [369,375].

Our study also differed methodologically from previous studies. Most used BMI as the main growth outcome [11,228,373,376,389], which can be difficult to interpret since it combines weight and height measures. For example, if antibiotics had a positive effect on linear growth, but no effect on weight gain, antibiotics would counterintuitively be associated with lower BMI despite the positive effect of antibiotics on height. The separate assessment of height and weight effects is an important strength of our study. Previous studies also commonly did not control for the illnesses that triggered antibiotic use [11,373,376], which may have resulted in estimates that were subject to residual confounding. We considered the indicating illness as an important confounder of the effect of antibiotics on growth, and correspondingly included multiple aspects of child sickness in the multivariable models. Finally, previous studies rarely reported the types of antibiotics given [11,373,376], which could have potentially differed from those given in our study population and may have different effects on growth. Inconsistency in the effect of antibiotics on weight gain has been documented in animal studies and suggests that many factors may play a role in the complicated relationship between antibiotic exposure and growth [24].

Strengths

Our study was based on rich longitudinal data and applied sophisticated statistical analyses to assess the effects of antibiotics on diarrhea and growth. Both the quantity and quality of data available for this study was high. The cohort provided highly detailed information about diarrhea incidence and severity such that presence of diarrhea and other symptoms was known

for each child on every day of follow-up. The recall period for diarrhea was short (3 days) such that recall bias was unlikely to affect the validity of our results. The proportion of missing values for covariates was low, less than 5% for baseline covariates. These data were high quality given the extensive quality control strategy including frequent retraining of field workers, regular instrument calibration and standardization of protocols, validation of information collected at home visits, monitoring of missing data, and double entry of data into the electronic database. In addition, the encouragement of study participants to visit the study clinic when ill allowed data linkage with clinic records to validate data from home visits and access non-diarrheal illness burden and treatments, which were important for antibiotic exposure classification and control of confounding. The availability of data from two previous cohorts allowed the comparison of Aim 1A results across studies from the same geographic area over time.

We used appropriate statistical analyses to account for analytic complications associated with longitudinal data, multiple diarrhea episodes per child, and repeated growth measurements. Previous studies have demonstrated the importance of modelling diarrhea longitudinally to describe the complicated relationships between age and repeated diarrhea episodes [25,26]. We accounted for changes in diarrhea incidence rates with age by analyzing closed cohorts in which all children were followed from birth to 3 years of age, and by adjusting for age flexibly with splines in analyses. Repeated diarrhea episodes required analysis methods that took into account clustering among observations within individuals. We used general estimating equations with robust variance estimators and bootstrapping to appropriately address correlation between episodes within children. We also appropriately handled time-varying exposures and confounders in Aim 1A, which changed for each diarrhea episode over the study period.

This study also involved developing and providing examples of relatively novel epidemiologic methods. In Aim 1A, we used inverse-probability weighted KM curves to estimate time differences and time ratios for the effect of antibiotic treatment of diarrhea on incidence of subsequent diarrhea episodes. The resulting estimates from this analysis were more interpretable than hazard ratios because they describe absolute differences in the timing of the subsequent episode between exposure groups. Because the majority of children experienced a subsequent diarrhea episode, an estimate of increased relative hazard from a Cox model would have been harder to conceptualize because an increase in hazard would not have necessarily translated to more children having diarrhea. We developed a SAS macro to perform this type of analysis more generally, which we have described in a manuscript in preparation for the *American Journal of Epidemiology*.

In Aim 1C, we used the parametric g-formula to estimate the impact of population-based interventions. This method has rarely been used to date, partly because it can be prohibitively complicated in the time-varying covariate setting. In contrast, our application of the parametric g-formula in a time-fixed setting was relatively straightforward to implement. By further increasing the visibility of this method, we hope more epidemiologists will find it an accessible analytic tool to estimate effects that are more relevant to public health.

Limitations

Because the parent studies were not designed to study antibiotic use, the assessment of antibiotic exposure information was suboptimal. Treatment was recorded systematically in the parent studies only for diarrhea, and quality of treatment information may have been variable since antibiotic types were recorded based on caregiver-report in a free-response question.

Dosage and duration of treatment were not recorded. In addition, we do not have information about prenatal antibiotic exposures or exposure through breastfeeding due to antibiotic treatment of the mother. Antibiotics are commonly given to mothers before Cesarean sections and at the beginning of labor if they are Group B *Streptococcus* positive [24]. However, we expect the effect of maternal antibiotic exposures on the infant to be minimal compared to direct exposures based on previous research [268,297,342].

While treatment information for non-diarrheal illnesses was not collected during routine field worker visits, prescriptions from clinic records were sufficient to classify children by exposure to any antibiotics regardless of clinical indication for treatment. We expect that almost all antibiotic exposures were captured in these clinic records since the study clinic was conveniently located in the residential area where study children lived and provided clinical care and medicines free of charge. We validated self-reported information by comparing antibiotic prescriptions for diarrhea from the clinic with self-reported antibiotic treatments from the cohort data. High concordance between caregiver-reported and antibiotic prescriptions for diarrhea supports our assumption that most antibiotic exposures were recorded in clinic records (78% of antibiotic prescriptions during diarrhea episodes were associated with caregiver-reported antibiotic treatment). Further, our results were consistent in sensitivity analyses when using alternative and more restrictive definitions of antibiotic exposure that required caregivers to report the name of the antibiotic given.

We were also unable to definitively characterize antibiotic treatment as necessary or unnecessary for Aim 1C analyses. Only information concerning the indicating illness was available, and other symptoms that may have indicated antibiotic treatment were unknown. Clinical criteria for diagnoses could have varied by physician at the study clinic, and some URI

and AGE cases could have been of bacterial etiology and responded to antibiotics. However, diagnostic capabilities in this area were not sufficient to distinguish between bacterial versus viral etiologies, such that treatment decisions must be made based on clinical signs alone. Because international guidelines do not recommend antibiotic treatment for the majority of cases of diarrhea, URI, and other AGE, classification of these treatments as not indicated was likely warranted.

The modified version of the Vesikari scale used in this study was designed to assess the severity of rotavirus diarrhea and has not been validated for all-cause diarrhea. However, this scale has been successfully applied to diarrhea due to *Cryptosporidium* previously in this population [402]. Increased severity of diarrhea episodes as indicated by the Vesikari scale was associated with higher prevalence of antibiotic treatment in the cohort, which was expected since more severe symptoms are likely to trigger greater care-seeking behavior. Given that a substantial proportion of diarrhea episodes were associated with rotavirus and the scale functioned as expected, we argue that severity measured using the Vesikari scale was appropriate when applied to all-cause diarrhea in this population.

As in any observational study, there was the potential for bias due to uncontrolled confounding in our study. However, the cohort had the advantage of detailed records of illness characteristics that were likely the main indications for treatment, and we adjusted for multiple components of disease severity. Because a clinical trial which randomized all antibiotic treatment in this setting would be unethical, we believe this evidence from a well-conducted prospective observational cohort study with good follow-up has a critical role in understanding the impact of antibiotics on diarrhea and growth. Also, by assessing antibiotic exposures given for common childhood illnesses in a realistic setting, our study may provide estimates of the

impact of antibiotics that are more generalizable to communities in LMICs when compared to trial results.

Finally, our results may not be generalizable to other populations since all three cohorts were sampled from the same source population in a fairly limited geographic area in southern India, which may not be comparable to other LMICs or even to the country of India as a whole. Treatment patterns, risk factors for diarrhea, and etiologic agents are variable across geographic sites and patient populations. Specifically, access to antibiotics varies based on local policy and law enforcement, and geographic variations in microbiota composition may affect the outcomes of antibiotic exposure. On the other hand, we do not expect the biological mechanism for the effects of antibiotics on diarrhea and growth to vary to such an extent that would preclude replication of our findings in other populations. Similar urban slum settings are common across Asia and Africa, such that results from this study likely also apply to other resource-poor settings.

Public health significance

This study is highly relevant to clinical policy and practice surrounding antibiotic treatment of diarrhea and other childhood illnesses. Because treatment of these illnesses is often not necessary, further evidence to inform treatment decisions will be useful for case management. Specifically, our evidence of harm to children treated with antibiotics early in life through increased diarrheal risk suggests that antibiotic exposure in the first 6 months of life should be limited where possible. In addition, because antibiotic treatment of diarrhea is associated with quicker onset of a subsequent episode, non-bloody diarrhea should generally not be treated with antibiotics unless other clinical signs or history indicates treatment would be appropriate.

We hope these recommendations will support efforts to improve rational antibiotic use. The overuse of antibiotics is a serious concern, and efforts to reduce overuse are a priority in the US and abroad. In September 2014, the White House published the "Report to the President on Combating Antibiotic Resistance" by the Council of Advisors on Science and Technology [462]. This report stressed the severity of antibiotic resistance as a public health threat and provided recommendations to maintain the effectiveness of antibiotics. Our results could support these efforts by providing physicians with evidence that antibiotics may be harmful, which would counter pressure from caregivers who demand antibiotics. In places where antibiotics can be purchased without a prescription, education efforts describing these harms may lead to a reduction in use for the treatment of illnesses for which antibiotics are not indicated.

We estimated the potential population-level impact of interventions with this aim in analysis 1C. These estimates are relevant to policy makers with interest in understanding the potential impact of policies to reduce antibiotic use. Direct effects on treated individuals would be only one component of a potential health policy decision, and our results concerning increased diarrheal risk contribute an important piece to the evidence base for constructing rational antibiotic use policies. Reduction of inappropriate antibiotic use at a large scale would benefit society in the long-term by reducing the prevalence of drug-resistant bacteria [393,394], preserving the efficacy of antibiotics for the treatment of serious human infections.

Finally, this study contributes epidemiologic evidence towards the rapidly growing area of research on the microbiota and its association with health and disease. Because our results are consistent with a potential biological mechanism that antibiotics can cause dysbiosis of the microbiota and increase susceptibility to diarrhea, the study indicates that future study of antibiotics in association with childhood development is warranted. Our results may inform

hypotheses for laboratory-based *in vitro* or animal studies to understand the complex relationships between microbiota diversity and composition, diarrhea, and growth in early childhood.

Future directions

Since our study provides the first evidence that antibiotic treatment may increase risk for future diarrhea, replication of our findings in other populations is needed. Specifically, it would be useful to assess these relationships in a setting with better assessment of antibiotic exposures. The study of Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health (MAL-ED) would be an appropriate choice given close follow-up of children and complete records of caregiver-reported antibiotic use [463]. This study was conducted in 8 countries and, similar to our study, involved birth cohorts followed longitudinally until 2 years of age. One of the study sites was based in Vellore in a similar source population as our cohorts (from a community across town). Replication of our analyses in these birth cohorts would allow validation of our findings in the same geographic area, and also indicate if the results are generalizable to children in other LMICs. Results from the current study and potential replication studies could be combined by incorporating the current results as Bayesian priors or including all results in a meta-analysis.

Our lack of evidence for an effect of antibiotics on growth was contrary to other recent studies which suggest antibiotics promote growth in children. Further investigation of the relationship between antibiotic exposure and growth is needed given the complex interactions between nutrition, illness, and child development that are further complicated in low-resource settings. Specifically, the potential competing mechanisms for an effect of antibiotics on growth

must be isolated to understand if either or both mechanisms (antibiotics as growth-promoters and antibiotics as causing future illness which hinders growth) are occurring. This may be most feasible in animal studies where conditions can be controlled and isolated. Future studies in human populations will also be needed to tease apart the potential pathways through which antibiotics could affect growth. Again, the MAL-ED study may provide a useful platform for these types of studies. Inclusion of the assessment of environmental enteropathy will likely be a key component to understanding the complex relationships between antibiotics and child development.

In collaboration with CMC, a follow-up study may be developed to assess the diversity and composition of the gut microbiota to better understand underlying biological mechanisms. The parent studies for this analysis included the collection and storage of a large number of stool samples for each study child, which were collected during every diarrhea episode (1-3 samples) and every 15 days regardless of illness. Sequencing of the microbiome in a subset of these stool samples may help explain how antibiotics affect diarrheal risk and growth. While many studies of the effects of antibiotics on the microbiota have been completed in mice and other animal models, studies in humans are more unusual and often involve a small number of adult subjects. Most studies are cross-sectional and compare the microbiotas in diarrhea cases with healthy controls [248,286,330,333]. For example, three studies from Vellore documented differences in microbiota composition in diarrhea cases [319,320,335], and acute diarrhea was associated with decreased microbiota diversity among children in Bangladesh [331,332]. However, because responses to antibiotics are individualized and influenced by prior exposure to antibiotics, aggregation of microbiota composition data across subjects may not be valid. Further, while studies have consistently documented changes in the microbiota during diarrhea, they do not

establish temporality. Based on fecal samples collected during the diarrhea episode alone, it is not clear if diarrhea causes the modifications in the microbiota, if dysbiosis of the microbiota is instead a risk factor for diarrhea, or if both processes are possible. Longitudinal comparison of samples taken from the same individuals before and after illness would allow comparison within-subjects, establish temporality, and hence provide results that will be more interpretable [17,286,340]. Therefore, the stool samples available in the parent studies provide an ideal resource for analyzing the effects of antibiotics on the microbiota in a longitudinal series of stool samples from the children before and after diarrhea episodes.

In the long-term, these results may contribute to the development of therapeutic and preventive interventions that could improve the resiliency of the microbiota against perturbations by antibiotics and reduce diarrheal risk. Interventions involving prebiotics or probiotics may stabilize and strengthen the microbiota in this way. Overall, a more complete understanding of the interactions between the microbiota, environmental enteropathy, and child development is needed to develop interventions to combat the long-term morbidity associated with childhood diarrhea.

Conclusions

While antibiotics are undeniably one of the most important public health discoveries in the last century, we are quickly learning that exposure to antibiotics has collateral effects both on individuals and at the population level. Antibiotics are commonly given during infancy at a time in which the developing gastrointestinal microbiota is most sensitive to perturbations. Concerns over the spread of antibiotic resistance have garnered the most attention by those promoting rational antibiotic use. However, explanations about the perils of antibiotic resistance are hard

for the public, and even clinicians, to conceptualize. An important outcome of this dissertation will be to highlight the impact of antibiotics on individual patients. By demonstrating that antibiotic treatment of diarrhea, and antibiotic exposure in general early in life, increases future rates of diarrhea, we provide an alternative and potentially more powerful argument for reducing antibiotic use. We counter the "at least they can't hurt" mentality towards antibiotics – antibiotics can cause sustained adverse effects among young children, and these effects should be considered when making treatment decisions for childhood illnesses.

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