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GUT IN FOCUS: EXTENDED ABSTRACT

Opportunities to assess factors contributing to the development of the intestinal microbiota in infants living in developing countries

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Recent evidence suggests that establishment of a healthy gut microbiota shortly after birth is important to achieve optimal growth and development of children. Being born into a resource-poor environment presents challenges to the establishment of a healthy gut microbial flora in the newborn. Among these challenges are births that occur at home, traditional pre-lacteal feeding of newborns leading to failure to initiate lactation, poor sanitation and water quality, early environmental exposure to, and infection with, enteric or other pathogens, suboptimal breast feeding duration and intensity, deficiencies in weaning and childhood diets contributing to micro- and macro-nutrient deficiencies, and the frequent use of antibiotics. These factors should be considered in the design and implementation of preventive and therapeutic interventions aimed at improving the health and development of these children.

Keywords: *probiotics*; *gut microbiota*; *environmental enteropathy*; *enteric infections*; *under-nutrition*; *child development*; *developing countries*

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It is now recognized that the human microbiota (microbial flora) and its collective genetic content (microbiome) play an important role in determining health status. The gut microbiota has been described as a 'microbial metabolic organ' because of its co-evolution with humans (1), its communication with other human organs including the brain (2), and because it contributes to health by metabolizing otherwise indigestible components of the diet (e.g. polysaccharides to short chain fatty acids) and synthesizing essential amino acids and vitamins and other bioactive compounds, by shaping a balance between pathogens and commensals, and by influencing the development of the innate and adaptive immune systems. Perturbation of the microbiota by infection with enteropathogens, biologic or chemical toxins, antibiotics, altered diet, and other environmental factors may give rise to dysfunction and diseases in the host. Recognizing the important role that the microbiota contribute to health has led to interventions aimed at restoring a healthy gut microbiota to prevent or treat disease. Fermented food-associated probiotics, fecal transplants from healthy individuals, and diets rich in the foods (prebiotics) that support a diverse and balanced intestinal microbiota are being evaluated as treatments for intestinal disorders such as *Clostridium difficile* colitis (3), inflammatory bowel disease (4), irritable bowel syndrome (5), diarrhea (6, 7), ulcerative colitis, and others (8).

Environmental enteropathy (EE), more recently referred to as environmental enteric dysfunction (EED) (9), is an ill-defined and difficult to diagnose intestinal pathology characterized by gut and systemic inflammation, altered villus architecture, alterations in gut barrier function, and absorptive capacity. It has been postulated that EED may develop when individuals live in a fecally contaminated environment where they are frequently exposed to enteric pathogens. It has also been postulated that EED may have a number of negative consequences, particularly in children under the age of two, including contributing to undernutrition, decreased growth velocity, stunting, depressed immune response to orally delivered vaccines, and impairments in cognitive development (10, 11). Unfortunately, these symptoms are slow to develop and thus difficult to recognize in their early stages. Improved methods for early diagnostics are essential. Current efforts

†See Appendix for list of investigators in the MAL-ED Network.

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are underway to identify improved biomarkers of gut inflamation, permeability, and decreased absorptive capacity that would identify children at risk of developing EED and its sequelae before they occur (12).

Approaches to prevent and treat EED in children are being considered (13, 14). These include improvement in water quality and sanitation (15), promotion of optimal breast feeding, improving the quality and diversity of diets, micronutrient supplementation of pregnant women and newborns, appropriate use of antibiotics, vaccination against enteric pathogens, and the use of pro- and prebiotics. The key question is – do we yet know enough to intervene effectively? It is likely that combinations of approaches, possibly varying from place to place based on the unique characteristics of the site, may have to be applied simultaneously to achieve maximal results. This forecast is based upon the fact the gut microbiota represents a variable dynamic human 'metabolic organ', which changes its structure and function from the time it is established shortly after birth. Factors such as age, diet, infectious diseases, medications, living environment, and many other variables may affect its composition (16, 17).

Subramanian et al. (16) describe a definable postnatal developmental program of assembly ('maturation') of the intestinal microbiota in children in Dhaka, Bangladesh, during the first two years of life while age-matched children from the same community with various forms of undernutrition exhibited relative microbiota immaturity. Treatment of children with severe acute undernutrition with ready-to-use therapeutic foods had only a temporary positive effect on the maturity of the microbiota. Either prolonged administration with existing therapeutic foods or new types of interventions may be needed to confer a lasting effect on the gut microbiota and prevent or reduce undernutrition and its persistent sequelae (stunting, cognitive deficits, and reduced immune response to certain vaccines). Kolling et al. (17) describe changes to the composition of the gut microbiota and the concurrence of pathogens that occur as humans age.

Plans and progress

The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) was started in 2009 to investigate the role of enteric infections, nutritional intake, and other environmental exposure variables on child development. It was designed as an observational, longitudinal, birth cohort study that followed approximately 200 children from birth to two years of age at each of the eight sites by the use of a harmonized common protocol and data collection forms. All participants consented, and then enrolled on a staggered schedule (10–12 per month over two years) to capture seasonal effects. Twice weekly home surveillance collected data on environmental factors, illness indicators (emphasis was placed on enteric infections), gut function biomarkers, nutrition, and anthropometry. Diarrheal stool samples were collected once during each episode and again at 14 days in the case of prolonged diarrhea episodes; normal, non-diarrheal surveillance stool was collected once per month for the duration of the study. All stool samples were analyzed to identify bacteria, viruses, and parasites known to produce enteric diseases. Study variables were examined for their contribution to child growth, immune response, and cognitive development. A more detailed overview of the MAL-ED project and the methodologies employed has been published (18). The study is being conducted at field sites in Iquitos, Peru (PEL); Fortaleza, Brazil (BRF); Venda, South Africa (SAV); Haydom, Tanzania (TZH); Vellore, India (INV); Naushero-Feroze, Pakistan (PKN); Bhaktapur, Nepal (NEB); and Dhaka, Bangladesh (BGD). These sites were known to have experienced high burdens of enteric diseases and growth deficits (stunting).

One of the study hypotheses of MAL-ED is that frequent enteric infection leads to EED, which could contribute to deficits in physical growth, cognitive development, and immune responses to expanded program on immunization (EPI) scheduled vaccines. As seen in Fig. 1, we observed that variable degrees of stunting (length for age Z score of at least two standard deviations below the World Health Organization (WHO) standard growth chart) did develop in MAL-ED cohort children during the first two years of life. Rates ranged from a few percent in BRF, to 20–40% in six of the other sites, to $>70\%$ in TZH. Even among those children who did not become stunted, most grew at a slower rate than would be expected under optimal conditions (data not shown).

The MAL-ED sites are representative of communities where effective sanitation is in short supply. Our study has revealed environmental conditions characteristic of such environments that could interfere with the establishment and maintenance of a healthy gut microbiota during the first two formative years and perhaps throughout early development. Among these factors are the high percentage of childbirths occurring at home in three of our sites; suboptimal breastfeeding (BF) and weaning diets; early exposure to, establishment of, and infection with enteric pathogens; and frequent use of antibiotics. These factors may conspire to create an environment that makes the use of probiotics to prevent or treat disease more difficult than in developed countries.

Childbirth at home

In five of the eight MAL-ED sites, there are relatively few home deliveries (Fig. 2). Most study sites are in close proximity to a medical facility (hospital or clinic) where improved delivery practices were available and where delivery by Cesarean section is uncommon.

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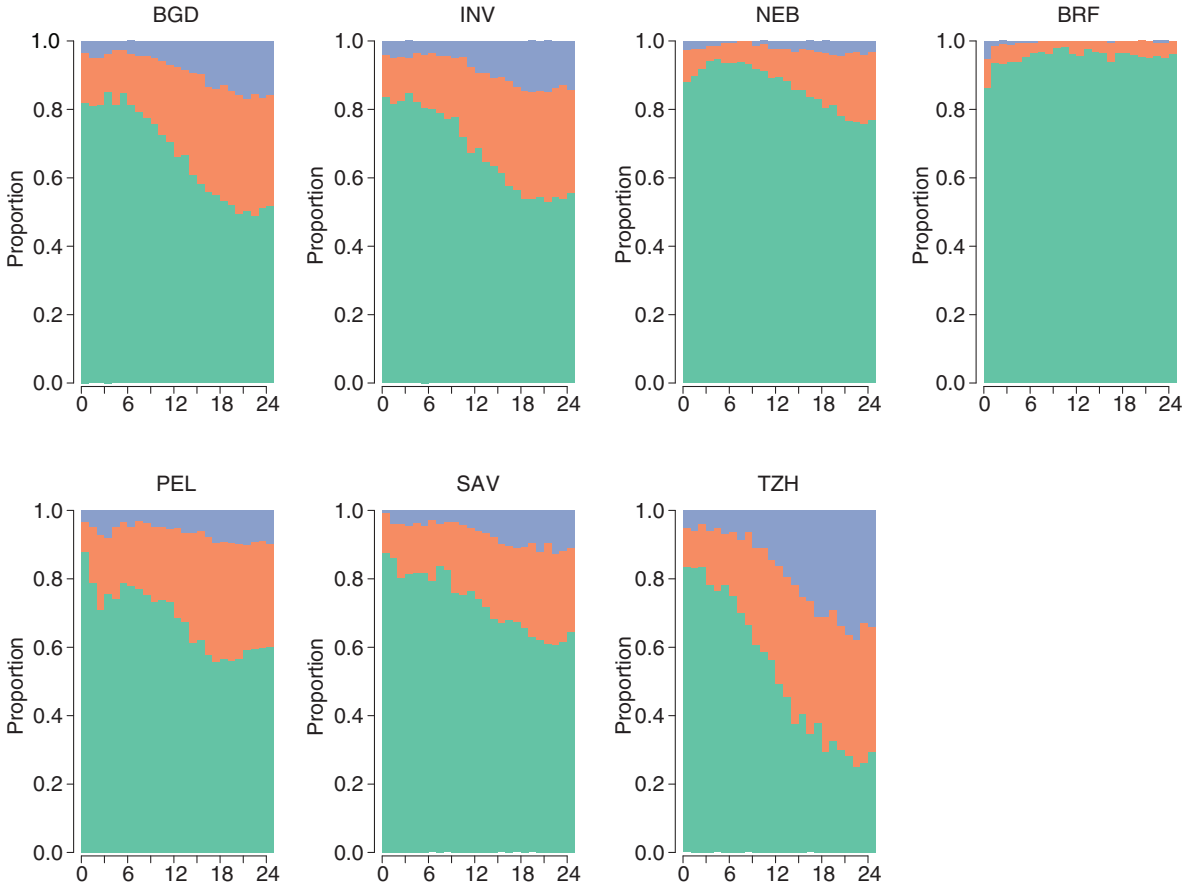

Fig. 1. Proportion of children stunted during the first two years at MAL-ED sites. Each child was measured every month for the first two years. Green – proportion of children not stunted (> -2 LAZ), Orange – proportion of children stunted (< -2, > -3 LAZ), Blue – proportion of children severely stunted (< -3 LAZ) at seven MAL-ED sites. Data pertaining to Pakistan are not available.

From a microbiologic perspective, vaginal delivery in an uncontaminated environment is preferred because it allows the microorganisms transferred from the mother's anal, vaginal, and skin microbiota to serve as the inoculum that seeds the gut ecosystem. However, at three study sites a significant percentage of births occurred at home [BGD (31%), PKN (59%), and TZH (50%)]. Home delivery in a contaminated environment has an assumed inherent risk of introducing pathogenic microbes to the infant's microbiota during or shortly after birth.

Suboptimal BF and weaning diets

The WHO recommends that the child be offered colostrum immediately after birth followed by six months of exclusive BF. None of the MAL-ED study sites achieved this goal. Figure 3 depicts survival curves for exclusive BF at each of the eight sites. The PKN site had the lowest rate (50% of children were no longer exclusively BF at 15 days of age) while BGD had the highest rate (50% still exclusively BF at about 110 days). However, additional data reveals that, while exclusive BF may be less than ideal, many children continue to receive predominant or partial BF for much longer. At BGD, more than 80% of the children were still receiving some breast milk in their diet at two years of age, while in INV, PKN, SAV, TZH, and PEL, less than 20% were. The introduction of other liquids and solids before six months of age increases the likelihood of pathogen exposure. Pre-lacteal feeding and early introduction of liquids or solids is the cultural norm in some of the MAL-ED sites and contribute to the rapid decline in exclusive BF observed in this study.

Methods we employed for dietary and micronutrient assessments have been described (19). Standardized dietary diversity indices were used to compare variation in diets across the sites during the first eight months of life. A varied diet is achieved by consuming four or more of seven different food groups during a 24 h recall period. All sites, with the exception of BRF, failed to reach a desirable diet diversity score. In addition, an adequate weaning and infant diet is needed to obtain required vitamins and micronutrients. The most common food groups consumed at many of the sites were grains, beans,

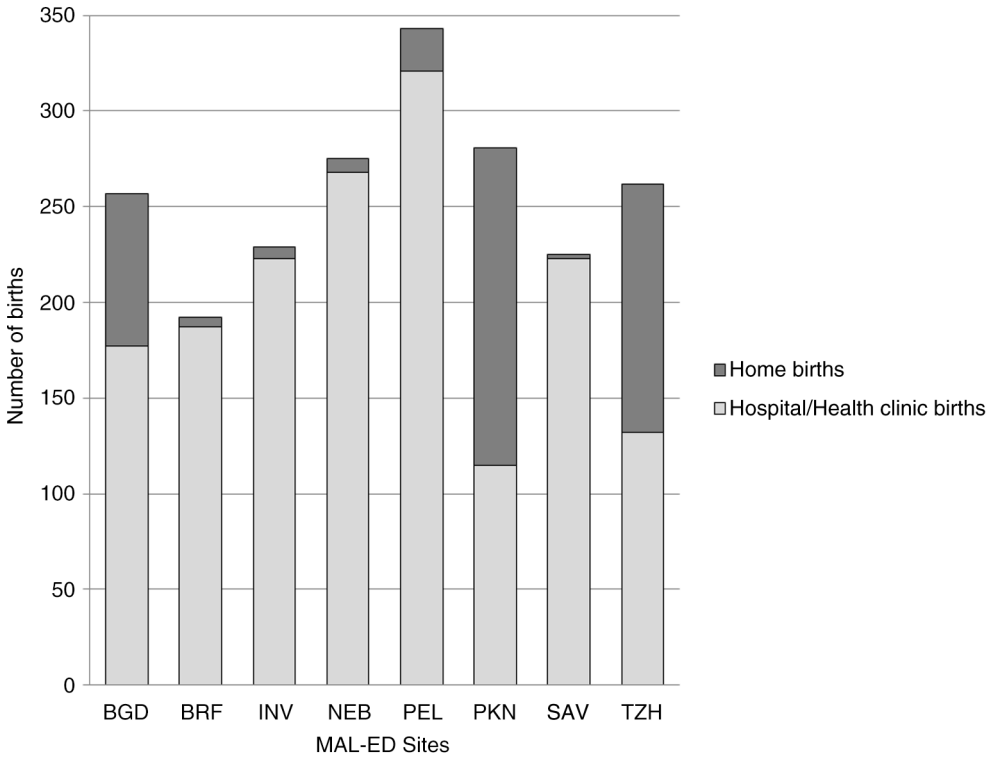

Fig. 2. The number of births occurring at home and at a medical facility in each of the MAL-ED sites.

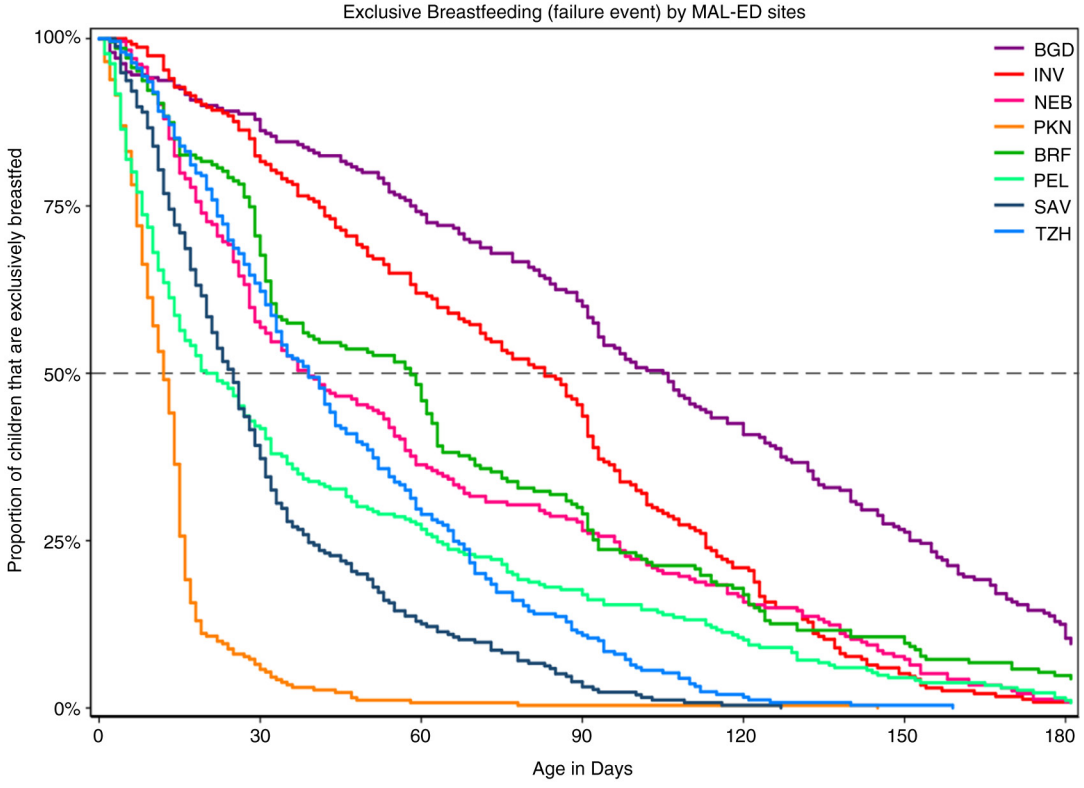

Fig. 3. Decrease in exclusive breastfeeding at MAL-ED Sites. Survival curves of exclusive breastfeeding are shown for each of the MAL-ED sites. Exclusive breastfeeding is defined as only having received colostrum and breast milk until such time as other liquids such as water, tea, solids are given.

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and dairy. Micronutrient deficiencies were observed in the MAL-ED cohort children. As an example, Table 1 shows the levels of anemia and zinc deficiency present at 7, 15, and 24 months of age (the times of blood draws) at each site.

Early exposure to, establishment of, and infection with enteric pathogens

At MAL-ED sites, newborn children are exposed to potential enteric pathogens early and often. As seen in Fig. 4a, in PKN, PEL, BGD, and TZH, about 50% of the children had potential pathogens (PP) present in normal stool at approximately one month of age. In INV, SAV, BRF, and NEB, that level was reached in two to three months. Virtually all infants had been colonized by PP at least once by the time they reached nine months of age. Most often, the presence of PP is not recognized because they produce no obvious symptoms. They were identified in this study because complete microbiologic analyses were conducted on these 'asymptomatic' normal stool samples that were collected once a month during the 24 months of active surveillance. That analysis detected enteric bacteria, parasites, and viruses and is described more completely in Ref. (20).

The first normal stool samples were collected in all cohort children one month after their birth. The most common PP identified in these samples are shown in Table 2. The most frequently identified pathogen at all sites was enteroaggregative *Escherichia coli* [EAEC, range: 8.4% (PEL) to 37.3% (PKN). *Campylobacter* was the second most common pathogen in six sites, and enterotoxigenic *E. coli* (ETEC) was in the top five pathogens at six sites (BGD, PKN, BRF, PEL, SAV, and TZH). Other common pathogens in these samples were *Cryptosporidium* and astrovirus. For a complete description of the microbiological findings from this study, see Platts-Mills et al. (Lancet, Global Health, in press).

While presence of enteric pathogens occurred very early at all sites, the age when the first diarrhea episodes occur in these children is quite varied (Fig. 4b). PKN is notable, in that diarrhea occurs at about the same time as the first detection of PP. At the other sites, the age at which 50% of the children have experienced their first diarrhea episode varies from about four months (BGD, INV, NEB, and PEL) to six months (TZH), 15 months (SAV), and 22 months (BRF). In these sites, particularly in SAV and BRF, many children never experience diarrheal symptoms despite the fact that they carry PP, often multiple pathogens simultaneously, during the first two years of their lives.

The high burden of PP does not appear to decrease as the children get older. As can be seen in Fig. 5, at least one enteric pathogen (bacteria, virus, or parasite) can be detected by culture, ELISA, PCR (for E. coli pathotypes), RT-PCR (for norovirus) or microscopy in 30–60% of non-diarrheal stool obtained from children at one month of age. This infectious burden increases throughout the observation period of the study and peaks at about 60% of normal stool in SAV, 70% in NEB, and 80–90% in the other six sites by the time the children are one year old. The frequency of isolation of pathogens from diarrhea stool is only slightly higher (peaks at 70–100% in all sites, data not shown). As noted above, many normal and diarrheal stools contain multiple pathogens simultaneously – as many as eight have been detected in normal stool (data not shown).

Use of antibiotics

The use of antibiotics to treat diarrhea and respiratory illness is high in many MAL-ED sites. As shown in Fig. 6a, the percentage of time during the first two years of life that children are treated with antibiotics for any reason

Table 1. Anemia and zinc deficiencies at 7, 15, and 24 months at MAL-ED sites

	Anemic[a]						Zinc deficient[b]					
	Number tested, % deficient						Number tested, % deficient					
	7		15		24		7		15		24	
BGD	202	49%	196	42%	175	27%	206	24%	195	19%	175	3%
PKN	261	72%	239	88%	223	83%	252	79%	238	69%	174	60%
INV	206	58%	228	56%	226	43%	221	51%	227	73%	224	88%
NEB	226	69%	220	50%	120	29%	221	33%	218	13%	118	23%
BRF	166	44%	150	40%	134	25%	147	4%	139	4%	81	2%
PEL	261	65%	227	51%	133	28%	233	2%	211	4%	104	0%
SAV	202	47%	227	53%	191	42%	30	7%	87	1%	44	7%
TZH	184	42%	197	40%	185	25%	132	29%	157	30%	150	27%

[a]Hb $<$110 g/L.
[b]Zn $<$9.9 mmol/L.

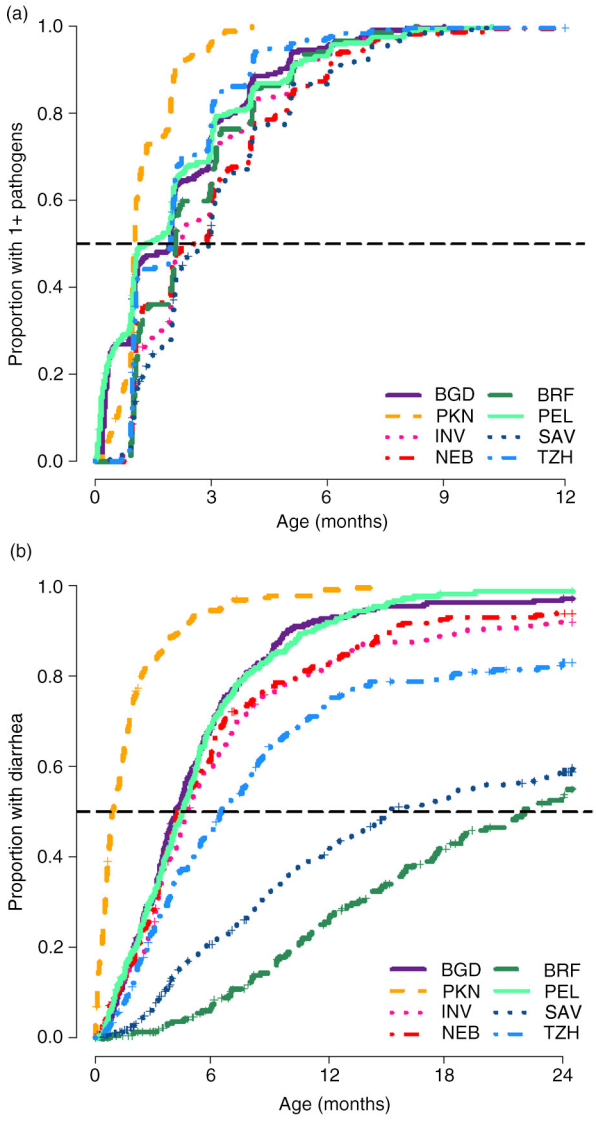

Fig. 4. (a) Proportion of cohort children at each MAL-ED site that have been infected with at least one enteric pathogen. (b) Proportion of cohort children at each MAL-ED site that has experienced at least one diarrhea episode.

ranges from a high of 16.9% in PKN to a low of 1.3% in BRF. Figure 6b shows that the number of diarrhea episodes for which antibiotics are given, also varies widely. In BGD, 60% of episodes are treated while in BRF only 11% are treated. Other sites fall between these extremes. Figure 6b also shows the average number of different antibiotics that are prescribed per episode of diarrhea by site. PKN used the highest number (average 0.9, range 0–8) while BRF used the lowest number of antibiotics (average 0.1, range 0–2). The most frequently prescribed class of antibiotic is metronidazole in PKN, NEB, and TZH; macrolides in BGD and PEL, cephalosporins in INV, penicillins in SAV, and sulfonamides in BRF.

Discussion

The MAL-ED study has identified factors that may contribute to the development of malnutrition and subsequent negative impact on a child's physical growth and cognitive development. These same factors may also hinder the use of probiotics as a way to establish or restore a beneficial microbiota to combat childhood malnutrition and to prevent or treat EED and its postulated negative effects. The study has revealed the need for renewed effort to educate mothers about the benefits of exclusive BF. In addition to providing the optimal early diet and defense against early diarrheal diseases, BF has been shown to have lasting positive effects on intelligence and educational achievement later in life (21), thus reinforcing the critical role that it plays in the establishment of a healthy microbiota and subsequent child development. None of the sites have achieved the WHO recommendation of exclusive BF for the first six months of life.

Diet has also been shown to affect the composition of the gut microbiota of children (22). In addition to providing the nutrients necessary for healthy growth and development, the weaning and infant diet should be thought of as contributing to the composition of a beneficial microbiota. The degree to which dietary supplements can be provided to support a healthy microbiota will be an important consideration in the design and implementation of trials aimed at testing the effectiveness of probiotic treatments.

While the use of antibiotics is justified in cases of serious bacterial infections, the use of narrow spectrum antibiotics could be used judiciously to target specific pathogen sensitivity and to minimize perturbations in the development of the microbiota that may have long-lived effects. The use of probiotics to treat EED or other intestinal disease should take into account the status of antibiotic use in the individual patient so as not to jeopardize the effectiveness of the probiotic microorganisms. The frequency at which antibiotics are used to treat enteric or respiratory infections in these settings will present a challenge to the effective implementation of probiotic therapies, especially if they will be used for prolonged periods of time. As shown recently by Rogawski et al., treatment of diarrhea in young children in India with antibiotics actually shortens the time to the next diarrheal episode by an average of eight weeks (23).

Petri et al. showed that the number of pathogens detected in either normal or diarrhea stool was about seven times higher in Bangladesh than in Virginia, USA, during the first year of life (13). This disproportionately heavy infectious burden borne by children in the developing world goes largely unrecognized as it occurs mostly in the absence of diarrheal symptoms. The questions remain as to the effects of these 'silent' infections on gut physiology and functions, including those associated with EED such as inflammation, leaky gut, and decreased absorptive capacity; what are the effects on the composition of the early microbiota?; should these pathogens be

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Table 2. Top five pathogens detected in non-diarrheal stools for one-month-old children in MAL-ED

	Sample size	1st pathogen (%)	2nd pathogen (%)	3rd pathogen (%)	4th pathogen (%)	5th pathogen (%)
BCD	241	EAEC (11.6)	Campylobacter (8.3)	Cryptosporidium (5.8)	ETEC (2.9)	Astrovirus (2.5)
PKN	185	EAEC (37.3)	Campylobacter (17.3)	Aeromonas (6.5)	ETEC (5.9)	Cryptosporidium (4.3)
INV	162	EAEC (17.3)	Campylobacter (3.7)	EPEC (1.9)	Astrovirus (1.2)	Rotavirus (1.2)
NEB	183	EAEC (17.5)	Campylobacter (7.7)	Astrovirus (4.9)	Cryptosporidium (4.9)	Atypical EPEC (2.2)
BRF	94	EAEC (27.7)	Cryptosporidium (12.8)	EIEC (9.6)	ETEC (7.4)	Atypical EPEC (6.4)
PEL	250	EAEC (8.4)	Campylobacter (7.6)	Cryptosporidium (6.0)	ETEC (3.2)	E. Histolytica (2.4)
SAV	214	EAEC (9.8)	Campylobacter (8.9)	ETEC (1.9)	Atypical EPEC (1.4)	Rotavirus (0.9)
TZH	239	EAEC (26.8)	Cryptosporidium (8.4)	Campylobacter (6.7)	ETEC (3.8)	Astrovirus (2.9)

Monthly, non-diarrheal stool is defined as a stool collected after 2 diarrhea-free days and preceding 2 diarrhea-free days. Only the first monthly stool is considered.
Table represents complete data only. All microbiology tests must have been performed for each sample to be included in the table.

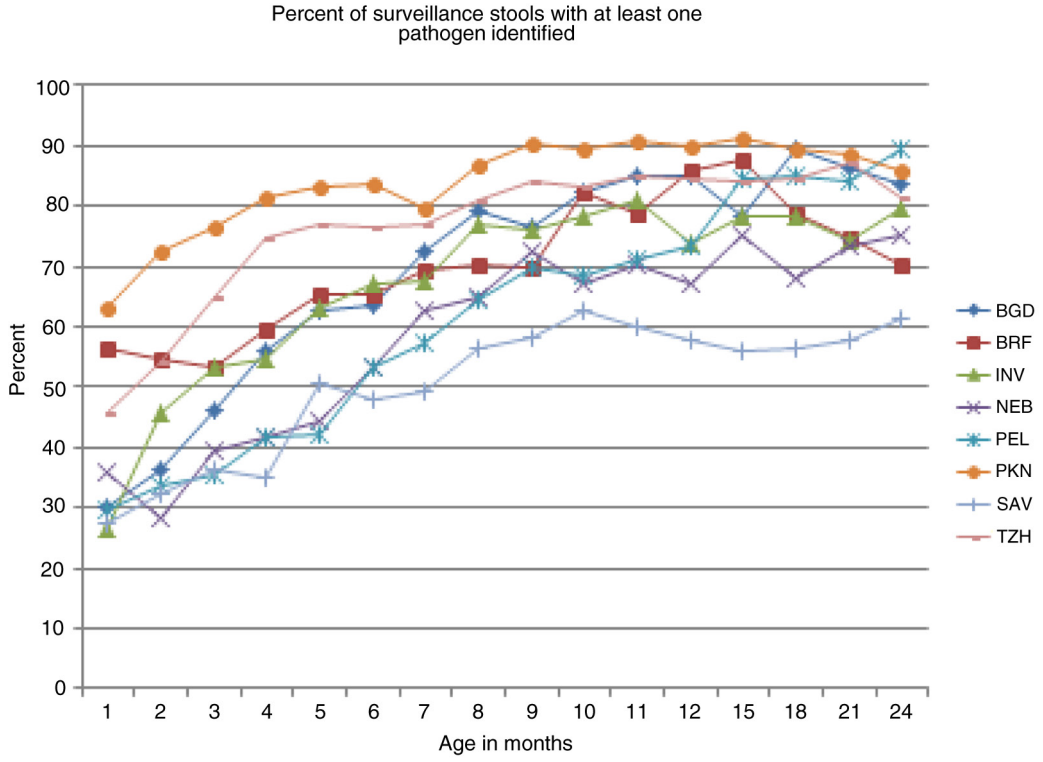

Fig. 5. Percent of normal stool samples containing at least one enteric pathogen. Normal stool samples were collected monthly and assayed for all enteric pathogens including bacteria, viruses, and parasites studied in MAL-ED. In the case of norovirus a subset of 10% of subjects were randomly selected from each site to have their normal stool samples assayed. The results for each site are shown as different colors indicated in the legend at the right of the figure.

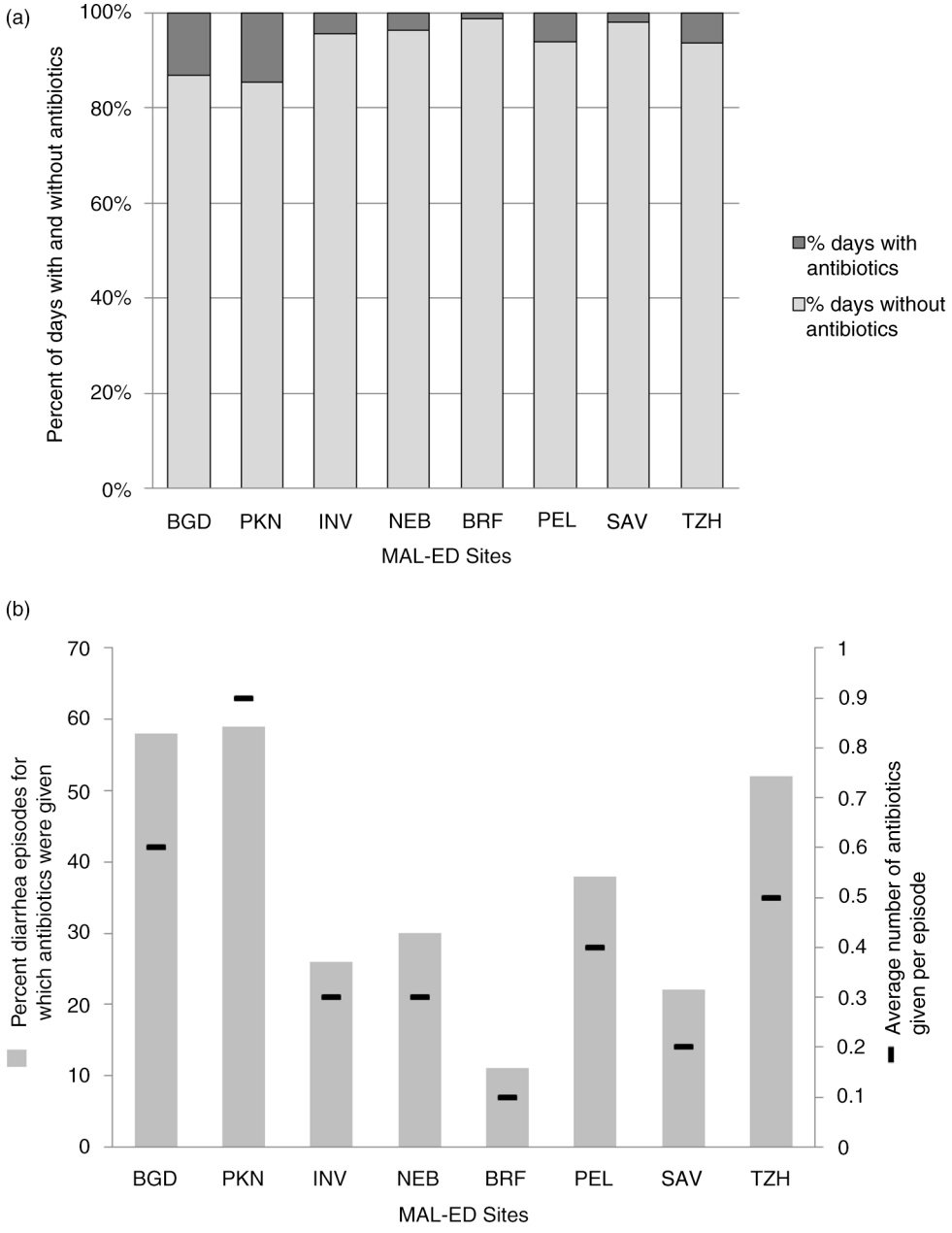

Fig. 6. (a) Percentage of days during the first two years of life that cohort children at each MAL-ED site received or did not receive antibiotics. (b) Treatment of diarrhea episodes with antibiotics at each MAL-ED site. Gray bars represent the percent of diarrhea episodes for which antibiotics were given. Black hash marks indicate the average number of antibiotics given.

considered as part of the 'normal' microbiota in areas of high fecal contamination?

It will be important to consider the relative extent of each these complicating factors at the sites where interventions will be tested. The MAL-ED data demonstrate the heterogeneity that exists between the sites. This heterogeneity will have to be considered in the design of a combination of interventions to target the specific conditions existing at each site.

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Conflict of interest and funding

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Appendix. MAL-ED Investigators and Institutional Affiliations

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