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Antibiotics and the developing infant gut microbiota and resistome

Molly K Gibson[1,4], Terence S Crofts[1,2,4] and Gautam Dantas[1,2,3]

The microbial communities colonizing the human gut are tremendously diverse and highly personal. The composition and function of the microbiota play important roles in human health and disease, and considerable research has focused on understanding the ecological forces shaping these communities. While it is clear that factors such as diet, genotype of the host, and environment influence the adult gut microbiota community composition, recent work has emphasized the importance of early-life assembly dynamics in both the immediate and long-term personalized nature of the gut microbiota. While the mature adult gut microbiota is believed to be relatively stable, the developing infant gut microbiota (IGM) is highly dynamic and prone to disruption by external factors, including antibiotic exposure. Studies have revealed both transient and persistent alterations to the adult gut microbiota community resulting from antibiotic treatment later in life. As antibiotics are routinely prescribed at a greater rate in the first years of life, the impact of these interventions on the developing IGM is emerging as a key research priority. In addition to understanding the impact of these disruptions on the infant gut microbial architecture and related host diseases, we need to understand the contribution of early life antibiotics to the selection of antibiotic resistance gene reservoirs in the microbiota, and their threat to successful treatment of infectious disease. Here we review the current understanding of the developmental progression of the IGM and the impact of antibiotic therapies on its composition and encoded reservoir of antibiotic resistance genes.

Addresses
[1] Center for Genome Sciences and Systems Biology, Washington University School of Medicine, St Louis, MO, USA
[2] Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, USA
[3] Department of Biomedical Engineering, Washington University, St Louis, MO, USA

Corresponding author: Dantas, Gautam (dantas@wustl.edu)

[4] These authors contributed equally to this work.

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Introduction

Antibiotics are the most prescribed medications in neonatal and pediatric populations in the United States [1–3]. In neonatal intensive care units (NICUs), ampicillin and gentamicin are prescribed twice as frequently as the next most common medication [2]. In children age 0–18, antibiotics are prescribed to more than 50% of individuals [1] and account for approximately 25% of prescriptions, with amoxicillin, azithromycin, and amoxicillin/clavulanate being the most common [3]. Antibiotic perturbation of the actively developing infant gut microbiota (IGM) has profound impacts on human health and disease throughout life, as alteration of the gut microbiota during this timeframe may disrupt metabolic and immune development [4••]. Equally important is the potential enrichment of the reservoir of antibiotic resistance genes ('resistome') available for transfer to pathogens [5], compromising treatment of infections in vulnerable populations. The phylogenetic and resistome composition of the IGM is connected, yet dynamic, with gut environment and antibiotic pressure increasing opportunities for horizontal gene transfer [6–8]. Until recently, the response of the IGM and its resistome to antibiotic perturbation was largely characterized by culture-based or PCR-based experiments [9•,10–12], which underestimate novel resistance genes. This response can be influenced by many factors, including antibiotic spectrum, duration, and delivery route (oral versus intravenous), as well as microbial community composition and antibiotic susceptibility. While it is clear that antibiotics disrupt the developing gut microbiota, eliminating taxa and enriching for antibiotic resistance genes (ARGs), we are just beginning to understand the relative contribution of each of these factors to the community-wide taxonomic and functional response to antibiotics.

Definitions and key concepts

Developmental progression: the normal patterned succession of bacterial species colonizing the infant gut in the absence of disruptive perturbation.
Antibiotic resistome: the collection of ARGs encoded in a microbial community.
Metagenomic functional selections: shotgun cloning and heterologous expression of microbial community DNA in model organisms to interrogate specific functions, for example, antibiotic resistance.
Preterm infant: infants born <33 weeks gestational age.
Very low birth weight infant: infants weighing <1500 g at birth.

Normal IGM and resistome development

The normal developmental progression of the IGM is patterned, yet highly dynamic and individual specific, and is shaped by many factors, including host physiology, genetics, diet, and environment [13,14••,15]. Upon birth, infants are exposed to a surge of microbes that colonize the epithelial surfaces, including the gastrointestinal system. The source and composition of this inoculating bacterial community is highly dependent on gestational age at time of delivery and, for term infants, mode of delivery [14••,16,17]. Term infants born vaginally are initially colonized by microbial communities resembling maternal vaginal microbiota (enriched in *Lactobacillus* and *Prevotella* spp.), while those delivered by caesarean section harbor communities that more closely resemble the skin microbiota (enriched in *Staphylococcus* and *Propionibacterium* spp.) [16]. For preterm infants (gestational age <33 weeks) the early gut microbiota composition resembles bacterial communities colonizing hospital surfaces and feeding and intubation tubing and are enriched in *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, and *Escherichia coli* [18•]. Mode of delivery in preterm infants does not appear to significantly affect the initial colonizing community and is instead hypothesized to be highly influenced by environment [18•,19]. Following initial colonization, term and preterm IGM alike begin to increase in diversity with continual dynamic turnover in bacterial composition driven primarily by chronological age; however, specific bacterial succession patterns are unique to these two populations [13,14••,19]. The most notable difference in succession patterns between infant populations includes an enrichment in Proteobacteria at <2 weeks in preterm infants. A detailed time series of a single term infant revealed the developing IGM is initially dominated by Firmicutes, with low levels of Proteobacterial species introduced in the first week of life and persisting as minor components (<10% relative abundance on average) throughout the first 2.5 years of life [20•]. By contrast, preterm IGM are quickly dominated by Proteobacterial species within the first week of life and maintain high levels, comprising on average >75% relative abundance of the community, throughout the first month [14••,21]. In healthy term infants there is a dramatic increase in *Bifidobacterium* and *Bacteroides* spp. within the first six months of life. By the end of the first year of life the IGM begins to resemble an adult-like microbiota, reaching full maturity by 2–3 years of age [13,15,20•]. It is still unclear if preterm infants eventually follow a similar developmental pattern once 'caught up' to term infants in postmenstrual age (gestational age plus chronological age) or if this population is set on a unique developmental trajectory.

The functional capacity encoded in the IGM also changes dramatically in the first year of life. In term infants, a shift is observed from lactose metabolism when diet is comprised of human milk and formula, to polysaccharide utilization upon the introduction of solid foods [20•]. While the gut-associated resistome comprises epidemiologically important functions, less is known about how this reservoir of genes develops in early life. Recent studies have shown that ARGs in the IGM are established within the first week of life, even in the absence of antibiotic exposure [22,23••,24,57]. Most investigations of the early resistome have employed culture-based or PCR-based methods [9•,10–12]. Focusing on readily culturable bacteria and previously identified ARGs vastly underestimates the diversity and abundance of ARGs in the gut microbiome [5]. To overcome these challenges, a recent study used culture-independent methods to characterize the gut resistome of 22 healthy infants and children aged one month to 19 years [23••]. Employing high-throughput functional metagenomic selections [25], the authors demonstrate that the healthy pediatric gut resistome is established early in life and persists throughout childhood. Of the 18 antibiotics investigated, only gentamicin demonstrated age-discrimination independent of antibiotic exposure with children >12 months of age harboring significantly higher levels of gentamicin resistance compared to younger children [23••].

Early-life antibiotics and the human microbiota

Preterm or very low birth weight infants are at highest risk for antibiotic associated perturbations, as they routinely receive empiric antibiotic therapy at birth [26,27•]. As with adults, short-term perturbations of the IGM follow soon after antibiotic treatment, the broad characteristics of which are known through culture-based methods [28]. Recently, culture-independent methods for interrogating microbial communities have emerged, relying on DNA amplification and sequencing. When applied to the developing IGM, some studies suggest both phylogenetic diversity and microbial load are depressed following antibiotic therapy. For example, 16S rRNA-based phylogenetic profiling of fecal microbiota from preterm infants receiving ampicillin and gentamicin during the first week of life had lower diversity compared to un-treated infants [27•]. However, another study comparing the fecal microbiota composition of infants treated with oral cephalexin to infants receiving no treatment did not reveal significant differences during the month following therapy [29]. These differing findings may be due to different antibiotic regimens, routes of antibiotic administration, choice of statistical analytical methods, or other uncontrolled factors. The difficulties inherent to untangling these variables informs both the need for large cohort studies of specific antibiotic regimens and studies in controlled animal models. Bacterial load is another measure found to decrease in some studies but not in others. Quantitative PCR of 16S rRNA has been used to estimate bacterial load in the gut. In unrelated studies examining the IGM following antibiotic therapy, bacterial load was found to be unaffected, slightly altered, profoundly decreased, or

even increased following treatment [15,30]. Again, the lack of a consensus may be due to uncontrollable variables inherent to infant cohorts.

Antibiotic treatment can also target specific phylogenetic subgroups of the IGM. Treatment of preterm infants with a variety of antibiotics, including penicillin, ampicillin, cephalexin, gentamicin, amikacin, erythromycin, vancomycin, clindamycin, and teichomycin, have been found to increase the percentage of potentially pathogenic Enterobacteriaceae while lowering the relative percentage of microbial taxa linked to a healthy microbiota such as Bifidobacteriaceae, Bacilli, and Lactobacillales spp. [27•,29,30]. In mice, reproducible effects on taxa have been noted. In mice exposed to subtherapeutic antibiotics through drinking water, no overall change in microbial load was detected, but a significant decrease in the ratio of Bacteroides to Firmicutes was observed [31]. In another study examining the consistency of phylogenetic responses to antibiotic perturbation, mice were treated with amoxicillin, metronidazole, bismuth, cefoperazone, and in combination. Under these conditions Proteobacteria, and in particular Enterobacteriaceae, dominated the intestines of the treated animals immediately after cessation of therapy, accounting for 73% of sequences. After two weeks without perturbation the microbiota of these animals returned to a low percentage Proteobacteria state (5.77%), though still higher than in untreated mice (1.2%). Treatment with cefoperazone, a broad-spectrum antibiotic, was in particular associated with loss of microbial diversity without recovery even six weeks post therapy [32]. In another study in which mice were administered either vancomycin or streptomycin in their drinking water, only vancomycin treatment was associated with significant reductions in both bacterial load and diversity, including depletion of Bacteroidales and marked enrichment of *Lactobacillus* spp. [33]. An important variable in several studies is the route of antibiotic administration. In many mouse studies, antibiotics are provided through the most facile means available, for example, through the animal's water supply or, in the case of infant mice, through the mother via milk [4••,31]. This is in stark contrast to antibiotic administration in the NICU, where the majority of antibiotics are provided through intravenous lines [14••]. A recent study in mice found significant differences when tetracycline or ampicillin were administered orally versus intravenously, highlighting the importance of this variable in evaluating the translational significance of murine model systems [34].

Long-term effects of early-life antibiotic therapy

Infants exposed to antibiotics during microbiota development may experience long-term disruptions. For example, disruptions have been noted at 90 days following treatment with a variety of antibiotics and three months after treatment with oral amoxicillin [30,35]. However, some studies have found no long-term microbial disruptions due to antibiotic use in human infants [15]. In mice, lack of recovery from antibiotic treatment at six weeks has been noted [32] and even subtherapeutic antibiotics have been found to have long-term effects on taxa associated with healthy microbiota such as *Lactobacillus* spp., Bifidobacteriaceae (decreased abundance) and Enterobacteriaceae (increased abundance) [4••,29].

Early-life antibiotic therapy has been linked to a variety of host outcomes and antibiotic-disrupted taxa have been linked causally to these as well. Broadly, antibiotic therapy can enrich for potentially pathogenic and antibiotic resistant Enterobacteriaceae, a bacterial family commonly resistant to beta-lactam antibiotics [27•,30]. Antibiotic therapy in infants has further been linked to increased risk of developing necrotizing enterocolitis (NEC), the leading cause of morbidity in NICU infants [36]. In one study of preterm infants, empiric antibiotic therapy lasting >5 days was associated with a significantly increased rate of sepsis, NEC, and death, with an attributable risk of 32 per 100 infants [26]. Another retrospective study of extremely low birth weight infants found that courses of antibiotics >5 days in the first days of life were statistically linked to increased risk of developing NEC and higher mortality rates. It was found that each additional day of antibiotic treatment increased the odds of an infant developing NEC by ~7% or developing NEC and dying by ~4% [37]. In these studies causative taxa were not identified. However, other studies have demonstrated loss of *Lactobacillus* and *Bifidobacterium* spp. and increased Enterobacteriaceae as a result of antibiotic treatment [4••,29]. Taxa from the Lactobacillaceae and Bifidobacteriaceae families have been linked to the prevention of poor outcomes in infants and are known to be important components of a healthy developing IGM and originate from the maternal microbiome [15,17]. Probiotic treatment of very low birth weight infants with *Lactobacillus acidolphilus* and *Bifidobacterium infantis* has been shown to reduce morbidity in these cohorts, as well as increase daily weight gain and decrease hospital stay times [38,39]. One potential mechanism of this protection is through interactions between the gut microbiota and the host immune system. Specific taxa, such as *Lactobacillus* spp., have been shown in model organisms to promote a healthy gut immune response and healthy modulation of the intestinal epithelial layer [40]. Perturbation of the maternal and IGM in a murine model was also found to modulate the levels of the IL-17 cytokine, leading to increased susceptibility to sepsis [41]. Outside of infancy, early life antibiotic use has also been linked to the development of other conditions later in life. Recent studies using a murine asthma model have found evidence implicating antibiotic-induced dysbiosis in increasing asthma rates later in life [33]. Similarly, antibiotics have been found to play a role in the induction of hypersensitivity pneumonitis [42]. Antibiotic treatment has also been linked to

obesity. Children exposed to antibiotics in the first six months of life were found to have a statistically significant increase in body mass. On the other hand, children treated with other medications or antibiotics after six months of life showed no such correlation [43]. In another study, antibiotic exposure during the first year of life was found to be associated with being overweight at age 12, with the association particularly strong in males [44]. Similar effects have been seen in mice under controlled conditions. In a pair of studies in which subtherapeutic antibiotics were administered to infant mice, treatment was found to induce metabolic changes in the host, including increased adiposity, modulation of liver mechanisms for cholesterol and lipid metabolism, and increased susceptibility to a high fat diet. Furthermore, these effects were directly linked to changes in the gut microbiota, including phylogenetic composition and metabolic function, and were found to transfer following administration of an altered microbiota to a healthy host [4••,31].

Enrichment of the infant antibiotic resistome

Significant alterations in the composition of the developing IGM in response to antibiotic treatment can cause a similar transformation in functional capacity, the most clinically relevant example being antibiotic resistance. When exposed to constant antibiotic challenge *in vitro*, microbial communities show evolution of multidrug resistance [45] as well as population-level resistance dynamics to antibiotic stress [46]. While the routes of evolution of antibiotic resistance and community-level dynamics are less well known *in vivo*, antibiotic therapy has been shown to select for survival of resistant members of the microbial community or for members capable of acquiring ARGs [47]. The persistence of these populations after cessation of therapy poses a long-term threat to the host as these populations can include potential pathogens as well as act as reservoirs for ARGs for transfer to pathogens [48,49]. For example, in a pair of studies in adults, treatment with clindamycin for seven days resulted in rapid development of resistant *Bacteroides* spp., with resistant clones constituting ~15% of the clones in the treated cohort compared to ~0% in the control cohort. This condition persisted through the entire 2-year study. Similarly, the macrolide resistance gene *ermF* was several logs higher in treated adults than in control and persisted for at least two years [11,50]. In another study, 1000-fold enrichment of the macrolide resistance gene *ermB* was found following treatment with clarithromycin and metronidazole, and was observed up to four years later even in the absence of additional antibiotic therapy [10]. While comparable studies on the effect of antibiotics on the IGM in early life are lacking, one similar culture-based study examined the oral microbiota of children treated with the antibiotic amoxicillin. Surprisingly, amoxicillin resistant bacteria were found both in children with and without drug treatment. In addition, approximately 50% of the amoxicillin resistant isolates also showed resistance to penicillin, with others also demonstrating resistance to erythromycin and tetracycline [35].

Notably, the route of antibiotic administration can strongly impact the emergence of resistant populations in the gut. Mice provided with an oral inoculum of either tetracycline or ampicillin resistant bacteria were administered each corresponding antibiotic either orally or intravenously. The expansion and contraction of the known resistance genes in the resistant bacteria were monitored by quantitative PCR. Oral administration of ampicillin was found to result in an approximate 4-log increase in ampicillin resistant gene copy number over intravenous administration, while the increase seen for oral administration of tetracycline was ~2-log. The difference in effect was hypothesized to be a result of how the host clears each antibiotic, with ampicillin being cleared solely through the urine and not interacting with the gut microbiota [34].

Conclusions

Given the exceedingly personalized nature of the human gut microbiota, we anticipate that highly sampled, longitudinal infant cohort studies combined with controlled mouse models of therapeutic levels of antibiotic treatment will begin to deconvolute the forces shaping these developing microbiota and their encoded ARGs. As we begin to understand more about the extent to which antibiotic resistance spreads within and between microbial ecosystems, there has been a concurrent increase in emphasis on addressing the challenge of antibiotic resistance from an ecological perspective [9•]. Importantly, this approach requires characterization of the overall abundance and diversity of ARGs in the environment and human-associated microbial communities [51,52]. Using culture-independent metagenomic and functional metagenomic techniques, recent studies have shown the human gut microbiota to be an extensive reservoir of ARGs [53,54], the abundance of which has been broadly correlated with antibiotic use practices by country [55•]. While a number of studies described above have demonstrated a significant response of specific drug-resistant strains or specific ARGs to antibiotic therapies using culture or PCR-based methods, the effect of antibiotics on community-wide antibiotic resistance remain unclear. Functional metagenomic studies of ARGs harbored in the guts of healthy infants reveal high potential for mobilization and overall disconnection between ARG and bacterial host [23••], suggesting a much more complicated relationship between the community composition and functional response to antibiotic resistance in the developing gut. Integration of culture-independent methods for community-wide investigation of corresponding community composition and functions, such as metagenomic functional selections [25] combined with marker or shotgun DNA sequencing [56], will be essential in filling in the current gaps in our system-wide

understanding of the effects of antibiotics on developing IGM and resistomes.

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