

Review

Oxygen and Metabolism: Digesting Determinants of Antibiotic Susceptibility in the Gut

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SUMMARY

Microbial metabolism is a major determinant of antibiotic susceptibility. Environmental conditions that modify metabolism, notably oxygen availability and redox potential, can directly fine-tune susceptibility to antibiotics. Despite this, relatively few studies have discussed these modifications within the gastrointestinal tract and their implication on *in vivo* drug activity and the off-target effects of antibiotics in the gut. In this review, we discuss the environmental and biogeographical complexity of the gastrointestinal tract in regard to oxygen availability and redox potential, addressing how the heterogeneity of gut microhabitats may modify antibiotic activity *in vivo*. We contextualize the current literature surrounding oxygen availability and antibiotic efficacy and discuss empirical treatments. We end by discussing predicted patterns of antibiotic activity in prominent microbiome taxa, given gut heterogeneity, oxygen availability, and polymicrobial interactions. We also propose additional work required to fully elucidate the role of oxygen metabolism on antibiotic susceptibility in the context of the gut.

INTRODUCTION

The microbiome refers to all the microorganisms that live on or within a well-defined environment and their collective genetic material (Lederberg and McCray 2001; Berg et al., 2020; Whipps 2019). Within the mammalian gastrointestinal tract, the microbial environment varies along its longitudinal and transverse axes such that microbes occupy distinct microhabitats and nutrient niches (Donaldson et al., 2016). Host lifestyle, diet, and age can modify these nutrient niches and impact the composition of the microbiome (Abubucker et al., 2012; Faith et al., 2013). In addition to nutritional content, oxygen gradients act as a selective pressure on the microflora so most microbes found in the lower gut are facultative or obligate anaerobes (Albenberg and Wu, 2014; Roediger 1980; Thursby and Juge 2017; Rowan-Nash et al., 2019).

It is well established that the gut microbiome plays a critical role in human development and health. Infants are first colonized when they pass through the birth canal and the composition of the child's gut microbiome starts to resemble that of an adult's within three years (Dominguez-Bello et al., 2010; Yatsunenko et al., 2012). It is important to note that some studies suggest the placenta or uterus has an established microbiome and may contribute to initial infant colonization *in utero* (Aagaard et al., 2014). However, this point is contentious because other work indicates that these are sterile environments and the detected bacterial reads result from contamination (Leiby et al., 2018; Lauder et al., 2016). In general, if developmental milestones are not met during the first few years of life, it has been proposed that the altered function and composition of the gut microbiota correlates with negative health outcomes (Langdon et al., 2016). For example, childhood asthma is associated with a reduction in gut microbiome diversity in the first 12 months of life (Abrahamsson et al., 2014). These negative consequences are not restricted to the developing microbiome and perturbation of the adult gut microflora has been implicated in numerous disease states including irritable bowel syndrome, obesity, chronic liver disease, neurological disorders, and colorectal cancer (Gophna et al., 2006; Manichanh et al., 2006; Walker et al., 2011; Turnbaugh, 2006; Garcovich et al., 2012; Henao-Mejia et al., 2012; Collins et al., 2012; Wu et al., 2013; Zhu et al., 2013). Given the gut microbiome's importance in host health, it is critical to discuss how the microbiome responds to perturbation (Rook and Brunet, 2005; Rook et al., 2013; Kondrashova et al., 2013; Koh et al., 2016).

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Perhaps the most dramatic of these perturbations is the administration of broad-spectrum antibiotics. Antibiotics significantly disrupt the human microbiome composition, diversity, and functional capacity (Cabral et al., 2019; Dethlefsen and Relman 2011; Lewis et al., 2015). Together, these result in reduced nutrient extraction and vitamin production and create an ecological niche that allows opportunistic pathogens to cause local and systemic infection (Guarner and Malagelada 2003; Blaser 2011; Dollive et al., 2013; Blaser and Falkow 2009; Chang et al., 2008). Furthermore, antibiotics can create and maintain selective pressure for drug resistant bacteria, thus establishing a reservoir for drug resistance (Maurice et al., 2014; Sommer and Dantas 2011; Penders et al., 2013). It is therefore critical to elucidate the effects of antibiotics on the host and the gut microbiome. However, we must remember the heterogeneity of the gut biogeography as we consider why some bacteria survive and others succumb to antibiotic pressure. This heterogeneity is likely a significant factor that impacts antibiotic efficacy in the human gut microbiome.

In order to investigate the efficacy of antibiotic treatment in an *in vivo* context, it is essential to first understand and distinguish between antibiotic resistance, tolerance, and persistence. All these terms concern the ability of cells to survive antimicrobial treatment. Resistance can be defined as the inherited ability of a population of microorganisms to grow in the presence of high concentrations of antibiotics, regardless of how long that exposure lasts (Brauner et al., 2016). Tolerance is a transient reduction in antimicrobial susceptibility induced by transcriptional or physiological changes in bacteria that facilitates survival of brief exposures to high concentrations of antibiotics (Brauner et al., 2016; Tuomanen et al., 1986). For example, biofilms often exhibit tolerance because of gradients in signaling molecules, nutrients, and other environmental factors (Cabral et al., 2018; Michiels et al., 2016). Lastly, persistence is the ability of a small subpopulation of bacteria, often dubbed “persisters” or “persister cells”, to survive antibiotic pressure by adopting a distinct phenotype from the primary population (Brauner et al., 2016; Cabral et al., 2018; Michiels et al., 2016).

Environmental conditions, such as access to oxygen and other nutrients, have been shown to affect metabolic activity, directly impacting tolerance and persistence behavior in bacterial populations (Amato and Brynildsen 2014; Borriello et al., 2004). Nutrient starvation can lead to increased tolerance and persister cell populations (Ghosh et al., 2011; Grant and Hung 2014; Li and Zhang 2007; Maisonneuve et al., 2013; Nguyen et al., 2011; Shah et al., 2006; Betts et al., 2002). Subsequently, the treatment of tolerant and persister cells can be improved by potentiating with central carbon metabolites and oxygen (Allison et al., 2011; Jensen et al., 2014; Meylan et al., 2017). Previous work has also found a transient upregulation of metabolic activity in response to antibiotic treatment and that bypassing metabolic efficiency contributes to tolerance (Lobritz et al., 2015; Meylan et al., 2017). Reactive oxygen species (ROS) are also commonly formed in response to antibiotic treatment and are a major cause of cell death after treatment, providing another facet through which oxygen can affect antibiotic susceptibility (Hong et al., 2019; Dwyer et al., 2015). The gut microbiome possesses a variety of zones with differing access to oxygen and nutrients; thus, understanding how oxygen and nutrient availability impact bacterial growth and antibiotic treatment is essential to defining how the microbiome responds to toxic perturbations.

In this review, we synthesize recent advances in our understanding of antimicrobial action, translating *in vitro* mechanisms to *in vivo* microbiome phenotypes. We first address the composition of the microbiome and how it is impacted by antibiotics. Next, we discuss the effects of oxygen and metabolism on antibiotic action *in vitro*. Finally, we integrate these points to make predictions of *in vivo* antibiotic spectrum and suggestions for future work.

THE MICROBIOME AND ANTIBIOTICS

The microbial habitats of the lower gastrointestinal tract vary significantly along its longitudinal and transverse axes. These microhabitats are shaped by gradients that create ecological niches and determine both microbial structure and function (Figure 1A). For example, oxygen (Zheng et al., 2015; Thermann et al., 1985; Kelly and Colgan 2016; Schwerdtfeger et al., 2019) and antimicrobial concentrations (Kim et al., 2018) decrease from the small intestine to the colon while bacterial load (Gu et al., 2013; Dieterich et al., 2018; Sender et al., 2016; Donaldson et al. 2016), pH (Nugent et al., 2001; Ilhan et al., 2017), and transit time (Donaldson et al., 2016) increase, typically selecting for facultative or obligate anaerobes (Roediger 1980; Thursby and Juge 2017). Facultative anaerobes that are bile acid tolerant, such as *Lactobacillaceae* and *Enterobacteriaceae*, constitute a majority of the microflora in the small intestine (Zoetendal et al., 2012;

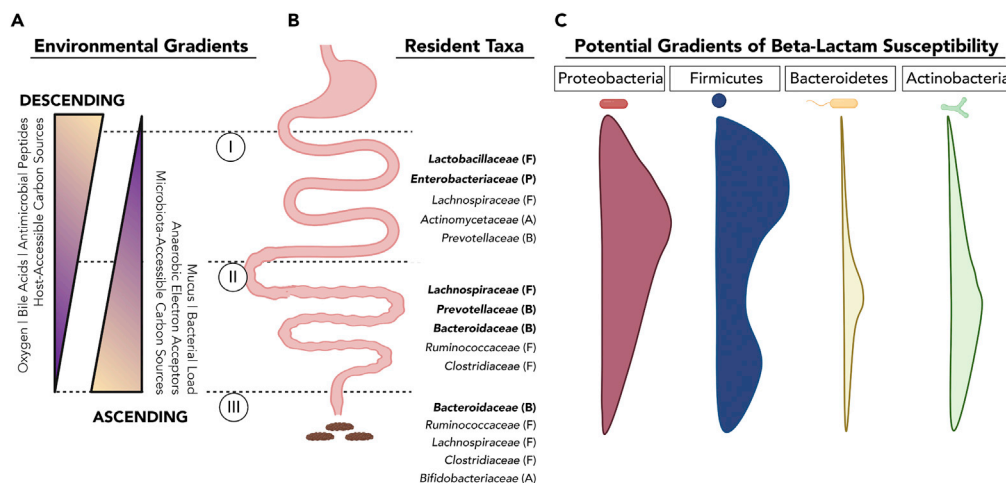


Figure 1. Characterizing Environmental and Microbial Drivers of Antibiotic Susceptibility within the Gastrointestinal Tract to Predict *in vivo* Antibiotic Activity

(A) The gastrointestinal tract is comprised of opposing gradients along the longitudinal axis. Factors such as oxygen, bile acids, antimicrobial peptides, and host-accessible carbon sources exhibit a descending gradient where they are most concentrated within the small intestine (I), have decreasing concentrations in the colon (II), and lowest effective concentration within the feces (III). Factors such as mucus, bacterial load, anaerobic electron acceptors, and microbiota-accessible carbon sources are most concentrated within the colon and least concentrated within the small intestine and feces.

(B) The resident taxa of the gastrointestinal tract vary along the longitudinal axis. The most prominent bacterial families within each intestinal region (I-III) are bolded, and the phylum designation is marked in parentheses (Bacteroidetes [B], Firmicutes [F], Proteobacteria [P], and Actinobacteria [A]).

(C) Potential gradients of beta-lactam susceptibility for the major phyla Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. Gradient predictions are based off a combination of environmental factors described in panel A and microbial composition described in panel B. Figure was created with [BioRender.com](https://www.biorender.com).

Gu et al., 2013). Fermentative anaerobes capable of utilizing undigested polysaccharides, such as *Prevotellaceae*, *Lachnospiraceae*, and *Bacteroidaceae*, dominate the large intestine (Figure 1B) (Gu et al., 2013; Donaldson et al., 2016; Thursby and Juge 2017). Importantly, many of these studies report relative abundance and not absolute abundance, and thus, the actual number of bacteria within these regions can change dramatically. For example, it is estimated that the bacterial load in the lower digestive tract can be orders of magnitude higher than in the small intestine (Sender et al., 2016).

The composition of the gut microbiome is in many ways directed by gradients set up within the host (Zheng et al., 2017). In return, the microbiome plays a central role in host metabolism and digestion, as well as immunological development and education that facilitates recognition of beneficial microbes (Forchielli and Walker 2005; Marchesi et al., 2007; Round and Mazmanian 2009; Rowan-Nash et al., 2019; Lathrop et al., 2011; Cebula et al., 2013; Nutsch et al., 2016; Russler-Germain et al., 2017). The gut microbiome also provides colonization resistance, in which beneficial microbes confer protection against potential pathogen colonization and any infections they might cause (Willemssen et al., 2003; Comelli et al., 2008; Johansson et al., 2008; Petersson et al., 2010; Kelly et al., 2015; Zheng et al., 2017). A disturbance of microbiome homeostasis (dysbiosis) is associated with various negative health consequences such as metabolic, developmental and immunological disorders that include inadequate nutrient and vitamin extraction, undernutrition, obesity, asthma, type 1 diabetes, and inflammatory bowel disease (IBD) (Turnbaugh, 2006; Sekirov et al., 2008; Abrahamsson et al., 2014; Azad et al., 2014; Guarner and Malagelada 2003; Marchesi et al., 2007; Hsiao et al., 2013; Stefka et al., 2014; Lewis et al., 2015). The high prevalence of autoimmune disorders and allergies in developed nations has been partially attributed to altered or reduced colonization of symbionts in early childhood, possibly induced by factors such as higher rates of caesarean section, more time spent indoors, a low-fiber Western diet, and increased use of antibiotics (Cabral et al., 2020; Rook and Brunet, 2005, 2010, 2012, Rook et al., 2013; Kondrashova et al., 2013). Given the widespread use of antibiotics (Shallcross and Davies 2014), it is essential to understand the mechanisms behind antibiotic-induced

microbiota disturbances in order to further appreciate the dramatic effects that gut perturbation has on human health.

Antibiotic-induced dysbiosis specifically decreases diversity of the gut microbiome and creates a nutrient niche vacuum that allows opportunistic pathogens to colonize and cause local infections (Dollive et al., 2013; Blaser and Falkow 2009; Chang et al., 2008). A notable example includes pseudomembranous colitis (i.e., antibiotic-associated diarrhea), a serious gut disease that can be induced by antibiotics. Pseudomembranous is caused by a rapid bloom in *Clostridioides difficile* after exposure to specific types of broad-spectrum antibiotics (Kelly et al., 1994; Blaser 2011; Aldeyab, 2012; Ley 2014; Theriot et al., 2014; Lessa et al., 2015; Vincent et al., 2016; Yoon and Yoon 2018; Chang et al., 2008). Dysbiosis can also prompt muciniphilic microbes to over-digest the colonic mucus layer in the absence of certain microbial metabolites, which increases an individual's susceptibility to pathogen infiltration and colonization (Ng et al., 2013; Desai et al., 2016).

The adverse consequences of antibiotic-induced gut dysbiosis are not only associated with the immediate off-target effects on the host microbiome but also with a wide array of long-term negative human health outcomes. For example, the effects of gut perturbation due to antibiotics have been proposed in chronic liver disease (Garcovich et al., 2012; Henao-Mejia et al., 2012), neurological disorders (Collins et al., 2012), and colorectal cancer (Wu et al., 2013; Zhu et al., 2013). Childhood asthma (Wickens et al., 2001; Kozyrskyj et al., 2007; Abrahamsson et al., 2014; Ni et al., 2019) and obesity (Azad et al., 2014) are also linked to antibiotic exposure *in utero* and in infancy. In particular, IBD is strongly associated with gut dysbiosis and antibiotic use (Card et al., 2004; Manichanh et al., 2006; Shaw et al., 2010, 2011; Vahedi et al., 2010; Hviid et al., 2011; Kronman et al., 2012; Schulfer et al., 2018), and it is becoming increasingly common in developing countries where antibiotics are being used with increased frequency. Taken together, these studies make it apparent that there are many adverse downstream effects of antibiotic use on the gut microbiome and human health.

Before we can fully understand the adverse effects of antibiotic administration, we must first acknowledge that there are intrinsic differences between drug classes. The first major distinction is between bacteriostatic and bactericidal antibiotics. Bacteriostatic drugs inhibit bacterial growth without killing the microbes while bactericidal drugs directly result in bacterial killing (Pankey and Sabath 2004; Ocampo et al., 2014). A second distinction must be drawn between narrow-spectrum and broad-spectrum antibiotics, which fall within the general classes of bacteriostatic and bactericidal drugs. Narrow-spectrum antibiotics are employed when the causative agent of infection is suspected, while broad-spectrum antibiotics are often used when the cause of infection is unknown or polymicrobial (Melander et al., 2018). Despite general applicability, broad-spectrum antibiotics can select for resistance across multiple species and can have negative off-target effects on the microbiome (Ory and Yow 1963). For example, fluoroquinolones are a class of antibiotics used to treat a variety of aerobic infections but their administration simultaneously decreases members of the *Enterobacteriaceae*, *Clostridiaceae*, and *Bifidobacteriaceae* families within the gut (Inagaki et al., 1992; Edlund et al., 1997). It is important to note that fluoroquinolones contribute to emergence of fluoroquinolone-resistant *Escherichia coli* (de Lastours et al., 2014; de Lastours and Fantin 2015; Bhalodi et al., 2019) while reducing the numbers of beneficial aerobic gram-negative species found in the gut (Edlund et al., 1997; Kourbeti et al., 2010). As with broad-spectrum therapies, narrow-spectrum antibiotics can also induce gut dysbiosis while clearing the causative agent(s) of infection. For example, the application of a narrow-spectrum antibiotic, such as fidaxomicin, can clear *C. difficile* infection (Golan and Epstein 2012; Zhanel et al., 2015). Unfortunately, fidaxomicin enacts its bactericidal effect against many commensal *Clostridia* species. This proves detrimental to the host because commensal *Clostridia* play a key role in short-chain fatty acid fermentation, colonization resistance, immunological tolerance, and cross-feeding within the gut (Rivera-Chávez et al., 2016; Pyrde et al., 2002; Golan and Epstein 2012; Lopetuso et al., 2013). Despite the profound differences in classes, drug-spectrums, and resistance rates, off-target antibiotic toxicity is a prevalent and unintended consequence of antibiotic exposure.

FACTORS THAT DICTATE ANTIBIOTIC ACTIVITY IN VITRO AND IN VIVO

In general, there are four main target processes for antibiotics: DNA replication, transcription, protein biosynthesis, and cell-envelope biogenesis. These are all essential biological processes with high energy demand and only subtle variation between species. However, it is in part this variation that drives differences in the spectrum of antibiotic activity. For a bacterium to be susceptible to a specific antibiotic, it must possess the primary binding target for that antibiotic. Because of target variation, not every bacterium

will be susceptible to every antibiotic. This is one of the main definers of the antibiotic spectrum, along with the incidence of resistance, tolerance, and persistence.

Resistance to antibiotics can come from a variety of mechanisms, generally considered intrinsic or acquired (Hollenbeck and Rice 2012; Reygaert, 2018). For example, Gram-negative bacteria can possess intrinsic resistance to a wide variety of antibiotics that gram-positive bacteria are susceptible to due to outer-membrane impermeability (Reygaert, 2018). Similarly, limiting drug import or increasing efflux (McMurry et al., 1980; Meylan et al., 2017) can provide significant resistances in otherwise susceptible bacteria. Resistance can also be acquired via changes to the primary target site, as is the case in methicillin-resistant *Staphylococcus aureus* where the *mecA* gene encodes a penicillin-binding protein (PBP), PBP2a, with significantly lower affinity for beta-lactam antibiotics (Hartman and Tomasz 2004; Centers for Disease Control and Prevention, 2013). Beta-lactamases, enzymes that destroy the amide bond in beta-lactams, are another common mechanism of resistance (Reygaert, 2018). Organisms that possess resistance genes can subsequently confer their resistance to other cells through either vertical or horizontal gene transfer (Thomas and Nielsen 2005; Rumbo et al., 2011). This ability to pass on advantages to other cells is a major defining characteristic of resistance. However, the acquisition of resistance genes is not inherently advantageous for the organisms that have them; their benefits often come with costs as they alter the function of important cellular processes. In a meta-analysis comparing the relative fitness of resistant organisms to the wild-type, Melnyk et al. found that most resistance mutations are linked to a fitness cost when not in the presence of antibiotics (Melnyk et al., 2014). The myriad of resistance mechanisms and consequences is masterfully reviewed by Munita et al. (Munita, 2016).

Whereas resistance is genetically encoded and transferable (Thomas and Nielsen 2005), tolerance is a transient and context-dependent phenotypic phenomenon. A well characterized example of a tolerance modulator is microbial metabolism, where slower metabolic rates have been shown to reduce antibiotic susceptibility (Lopatkin et al., 2019). Because antibiotics often target energy-consuming processes, reduced metabolism allows bacteria to survive transient antibiotic exposure by limiting the activity of the target processes (Cabral et al., 2018; Conlon et al., 2016; Fung et al., 2010). Conversely, permissive metabolic states are associated with increased susceptibility, and antibiotics themselves can cause an increase in the activity of target processes (Adolfson and Brynildsen 2015). This leads to a phenomenon called “futile cycling”, where cells continue the activity of an incapacitated process, consuming ATP and other essential resources (Cho et al., 2014; Adolfson and Brynildsen 2015). This can be seen in the action of beta-lactam antibiotics, where the inactivation of PBPs disrupts the cross-linking of peptidoglycans (PGs) in cell-wall biosynthesis (Cho et al., 2014). Although this disrupts cell-wall formation, the cell continues to generate PGs, using up ATP and precursor materials in the process (Cho et al., 2014). This continuous use of ATP by a useless process demands increased respiration, placing another burden on the cell. Indeed, separate work has shown that bactericidal antibiotic activity, including that of beta-lactams, leads to an increase in bacterial respiration (Belenky et al., 2015). Futile cycling has also been implicated in increased persister cell susceptibility to antibiotics (Mok et al., 2015) and higher sensitivity to oxidative stress (Adolfson and Brynildsen 2015).

While some work has shown that a higher growth rate is directly correlated to increased antibiotic efficacy (Greulich et al., 2015; Lee et al., 2018), recent work has indicated that this phenomenon is better attributed to increased metabolic activity (Lopatkin et al., 2019). These statements do not necessarily contradict one another, as increased growth rate necessitates higher metabolic activity (Lipson 2015), but rather clarifies metabolism as a better lens to view this through. Poor environmental conditions, such as limited access to nutrients (Nguyen et al., 2011) and oxygen (Borriello et al., 2004; Walters et al., 2003), have been shown to reduce metabolic rate and decrease susceptibility to antibiotics (Dwyer et al., 2014). Anoxygenic and otherwise nutrient-poor conditions are especially prevalent in biofilms, where there exist disparities in access to metabolites and oxygen throughout the structure (Michiels et al., 2016). The center of a biofilm suffers from poorer access to the environment as well as decreased flow of nutrients and waste compared to the outer edges of the biofilm. Because of this, cells in the center of biofilms tend to possess lower metabolic activity, and therefore higher tolerance to antibiotics, than the cells that live in the periphery (Walters et al., 2003; Williamson et al., 2012). Additionally, stress response systems, such as the SOS system, have been shown to induce tolerance in biofilms in response to external stress such as starvation (Bernier et al., 2013). A parallel can be drawn between biofilms and the environmental context of the gastrointestinal tract. The gut also possesses local and longitudinal differences in metabolite and especially oxygen availability, which serve to alter the behavior and susceptibility of the bacteria within the microbiome depending on the conditions surrounding them (Zheng et al., 2017).

The gut environment may also impact persister cell formation. Persister cells occur as a subpopulation, between 0.1% and 1%, of the major population (Lewis, 2010). These cells adopt a separate phenotype that results in drastically reduced metabolic activity that borders on dormancy, allowing for survival in high concentrations of antibiotics for extended periods of time (Cabral et al., 2018; Keren et al., 2004; Amato et al., 2013). These cells are able to survive treatment and repopulate an infection after eradication of the major population, leading to chronic infections (Grant and Hung, 2014). The next population, however, does not possess the same phenotype as their parents, but rather a similar heterogeneous phenotype distribution as before (Bigger, 1944). The repopulation of an infection after seemingly successful therapy is often described as the key clinical significance of persisters. They contribute to the severity of multiple bacterial infection types including *Pseudomonas aeruginosa* lung infections (Mulcahy et al., 2010), tuberculosis (McCune et al., 1966a; 1966b; Zhang et al., 2012), and recalcitrant *Staphylococcus aureus* endocarditis and bacteremia (Kim et al., 2018; Mikkaichi et al., 2019). Likewise, *Salmonella enterica* Typhimurium (*S.* Typhimurium) persister cells were found to undermine host defenses by releasing virulence factors that altered the cytokine profile of infected macrophages so that they entered an infection-permissive and non-inflammatory state, enabling the pathogen to bloom once antibiotic treatments ceased (Stapels et al., 2018). *S.* Typhimurium persister cells also promote the spread of resistance plasmids within the human gut (Bakkeren et al., 2019). Taken together, it is clear that persister cells pose a significant threat to human health because they can directly modulate both the gut microenvironment and host immune responses, in addition to promoting the spread of resistance and directly contributing to chronic and relapsing infections.

The formation of persister cells does not have one root cause or explanation, but rather appears to be a process with multiple entry points (Grant and Hung 2014), such as through the stress response (Dörr et al., 2010; Ghosh et al., 2011). Additionally, reduced metabolite availability has consistently been shown to induce persister cell formation through ATP depletion (Conlon et al., 2016; Kwan et al., 2013), amino acid limitation (Christensen-Dalsgaard et al., 2010), and nutrient starvation (Amato and Brynildsen 2014; Gao et al., 2010; Li and Zhang 2007; Betts et al., 2002). These work together to suggest metabolite limitation is a major cause of persister cell formation. Biofilms have also been implicated in the formation of persister cells (Michiels et al., 2016), as they can possess nutrient limiting conditions that promote persister development. Relevantly, high respiratory activity during stationary phase has been associated with high redox activity, while lower respiratory activity, and therefore lower redox activity, during stationary phase is an indicator of “healthy” growth in the future (Orman and Brynildsen, 2015). When provided with fresh nutrients, cells with lower redox activity are able to quickly transition to growth, while cells with higher redox activity transition poorly, and instead tend toward persistence (Orman and Brynildsen, 2015).

With both persistence and tolerance, a high rate of catabolic metabolism is a predictor of susceptibility. The availability of terminal electron acceptors in both aerobic and anaerobic bacteria is an important determinant of metabolic rate, ATP generation, and a switch between respiration and fermentation (Conlon et al., 2016; Gussarov et al., 2009; Lin and Iuchi 1991; Lobritz et al., 2015; Shan et al., 2017; Spiro and Guest 1991; Uden et al., 1994). Thus, as we consider antibiotic action, the oxygen content or the reduction potential encountered by bacteria must also be considered. In bacteria undergoing bactericidal stress, oxygen availability has two negative consequences. First, abundant oxygen provides the capacity to drive toxic futile cycles through the generation of ATP (Adolfson and Brynildsen 2015; Cho et al., 2014), and second, the byproduct of this elevated metabolism is the production of ROS (Hong et al., 2019). The rapid overproduction of ROS damages cellular components and leads to further exacerbation of futile cycles and ROS production (Cho et al., 2014; Hong et al., 2019; Belenky et al., 2015). This ROS-driven mechanism of antibiotic action was proposed in the mid-2000s (Kohanski et al., 2007; Wang and Zhao 2009), and has been supported by a large body of work (Dwyer et al., 2014; Grant et al., 2012; Jensen et al., 2014; Van Acker and Coenye, 2017; Wang and Zhao 2009; Zhao et al., 2015; Luan et al., 2018; Foti et al., 2012). However, some results have contradicted this finding (Keren et al., 2013; Liu and Imlay 2013), leading to significant discourse in the field. While it is beyond the scope of this review, several others have summarized the contribution of ROS to antibiotic lethality (Drlica and Zhao, 2020)(Lam et al., 2020). While ROS toxicity clearly plays a vital role in antibiotic-induced death, the primary target of the antibiotic is the essential trigger initiating the toxic cascade and the primary target may also be the main contributor to toxicity, depending on antibiotic concentration and the conditions encountered by the bacteria. For instance, at high concentrations, beta-lactam antibiotics can kill by inducing cellular rupture or at lower drug concentrations by a combination of cell-wall damage, futile cycles, and ROS generation (Cho et al., 2014; Lam et al., 2020). It is thus likely that the contribution of ROS to toxicity is dependent on the metabolic environment and respiratory capacity of the bacteria.

From a metabolic perspective, oxygen levels and redox potential can directly impact the capacity of the electron transport chain (ETC), proton motive force (PMF) generation, and ATP synthesis. At lower redox potentials, alternate terminal electron acceptors reduce the efficiency of the ETC, subsequently negatively affecting PMF. This could lead to reduced uptake of antibiotics such as aminoglycosides, whose transport is coupled to PMF activity (Eswaran et al., 2004) (Taber et al., 1987) and have been shown to have significantly lower uptake in metabolically reduced states like persistence (Allison et al., 2011; Meylan et al., 2017). As has been discussed previously, limiting metabolism can attenuate the efficacy of antibiotic treatments. Oxygen is the terminal electron acceptor for the aerobic ETC that drives bacterial respiration. As oxygen becomes limited, redox potential lowers, the ETC becomes less efficient, and bacteria are forced to utilize different forms of ETCs with different terminal electron acceptors in order to produce ATP (Gun-salus, 1992). As the redox potential lowers, the cell shifts away from respiration toward fermentation, and both ATP generation and activity are less efficient. Ultimately, this may lead to lower susceptibility because as cell metabolism and antibiotic uptake slow down, the negative effects of futile cycling and the activity of antibiotic target processes are minimized (Van Acker and Coenye, 2017).

The links between oxygen content, aerobic metabolism, and antibiotic efficacy have been demonstrated repeatedly. For instance, exposure to ROS can increase the rate of persister cell death (Grant et al., 2012). Treatment of biofilm infections can be improved by hyperbaric oxygen therapy (Jensen, 2019), whereas oxygen limitation is a key determinant in their tolerance (Borriello et al., 2004). It is well documented that aerobic metabolism leads to higher susceptibility when compared to anaerobic metabolism, supported by findings that increased metabolic rate leads to higher antibiotic susceptibility as discussed above. When considering all of these factors together, it becomes clear that oxygen availability plays a significant role in the effects of antibiotics on the target cell and must be considered when evaluating treatment in the context of the gut.

While a large body of work shows the *in vitro* effects of the metabolic environment on antibiotic susceptibility, understanding the *in vivo* conditions found in the patient are necessary to paint the whole picture of antibiotic efficacy. A prime example of this comes from *Burkholderia pseudomallei*, the aerobic Gram-negative bacterium responsible for melioidosis, a dangerous disease that often manifests through the formation of organ abscesses (Cheng and Currie, 2005). Although there are established antibiotic regimens to treat melioidosis (Chaowagul 2000; Jenney et al., 2001; White et al., 1989), they can last up to 5 months, with relapse occurring between 13 and 23% of the time (Cheng and Currie, 2005). Hamad et al. sought to investigate the effects of anoxia on the antibiotic susceptibility of *B. pseudomallei* since the prolonged and often unsuccessful treatments are associated with the formation of anoxic abscesses and the ability of *B. pseudomallei* to utilize an alternative anaerobic metabolism. They found that anoxic conditions such as those present in abscesses promoted the development of a population that was highly tolerant to the antibiotics conventionally used to treat melioidosis, but that nitroimidazoles, which are usually used to treat anaerobic infections, were effective. Additionally, they observed the development of incredibly long-term persisters with the ability to survive at least one year (Hamad et al., 2011). This subpopulation was entirely tolerant not only to aerobically effective antibiotics, but also to nitroimidazoles, and can help explain the frequent relapse of infections (Hamad et al., 2011). By matching their *in vitro* model more closely to the *in vivo* conditions, Hamad et al. were able to elucidate anaerobic metabolism as a probable cause for the difficulties associated with treating melioidosis.

Biofilms are another prime example of how limited oxygen availability can lead to anaerobic metabolism and subsequent antibiotic tolerance. The ability of bacteria to form biofilms is incredibly common, with prevalent pathogenic examples including *B. pseudomallei*, *P. aeruginosa*, and *S. aureus*. These biofilms often develop anoxic zones (Stewart et al., 2016; Walters et al., 2003), which promote the utilization of anaerobic metabolic pathways for the cells within. This has been shown to be a major contributor to biofilm tolerance to antibiotics (Walters et al., 2003). Developing effective treatments for biofilm infections is incredibly important to human health as, according to the NIH, around 80% of chronic infections are caused by biofilms (Davies 2003). These infections can be over 1000 x more resistant to antibiotic treatment than their planktonic counterparts (Soriano et al., 2009). Additionally, metabolite-limiting conditions within biofilms typically lead to the formation of populations of persister cells (Amato and Brynildsen 2014; Bernier et al., 2013; Betts et al., 2002), contributing to their ability to resume infection after treatment ceases. These characteristics can make biofilms notoriously difficult to treat and can also be exacerbated by host conditions. For example, biofilm infections are incredibly common in patients with cystic fibrosis (CF), where a

genetic defect in chlorine pumps leads to buildups of thick mucus on mucoidal tissue. The conditions that arise from CF are beneficial for opportunistic bacteria such as *P. aeruginosa* to establish themselves in a biofilm infection (Baltimore et al., 1989; Lam et al., 1980; Worlitzsch et al., 2002). These biofilm infections, particularly *P. aeruginosa*, are incredibly dangerous to patients with CF, and while treatments are improving, most current estimates place the median life expectancy of patients with CF in the low-mid 40s. Since oxygen limitation is a major contributor to antibiotic tolerance in biofilms, several studies have demonstrated antibiotic treatments to be improved by supplementing with hyperbaric oxygen therapy in order to induce aerobic metabolism and increase ROS production (Jensen, 2019; Kolpen et al., 2016, Kolpen et al., 2017; Møller et al., 2019; Lerche et al., 2017). Because these *P. aeruginosa* biofilm infections establish themselves within the anoxic mucus (Worlitzsch et al., 2002), these studies sought to imitate that environment by embedding *P. aeruginosa* aggregates in agarose. In particular, Møller et al. demonstrated an increased efficacy of tobramycin when supplemented with hyperbaric oxygen therapy, and Kolpen et al. demonstrated the beneficial effects of this on ciprofloxacin action (Møller et al., 2019; Kolpen et al., 2016). Recent work has suggested that biofilm-like structures form in the mucus layer of the gut (Duncan et al., 2020). Therefore, similar conditions affecting biofilm susceptibility in other types of mucus-associated infections may also play a role in antimicrobial susceptibility in the gut.

While antibiotic action is impacted by the metabolic conditions encountered by the bacterium, antibiotics can also perturb the conditions of the gut from a host perspective. For example, inflammation that results from antibiotic-induced dysbiosis has been shown to promote anaerobic metabolism in pathogens such as *S. enterica* by increasing availability of certain terminal electron acceptors (Spiga et al., 2017; Winter et al., 2010). The inflammatory response is a multifaceted beneficial process for metabolically flexible pathogens like *S. enterica*. Lopez et al. demonstrated that *S. enterica* can utilize host-derived nitrate from inflammation to drive anaerobic respiration and growth within the cecal lumen, an environment where oxygen concentration is typically too low to permit respiratory metabolism (Lopez et al., 2015). Additionally, Rivera-Chávez et al. showed how the perturbation of the gut microbiome through antibiotic action can deplete the gut of butyrate. Since butyrate consumption by colonocytes is essential for maintenance of anaerobicity, this butyrate reduction allows *S. enterica* to employ aerobic metabolism in the gut, leading to growth (Rivera-Chávez et al., 2016). Yang et al. recently found tissue-specific alterations in metabolite availability after antibiotic treatment, suggesting that these changes are not necessarily microbiome-specific but may instead be a synergistic result of local host cell, pathogen, and symbiont activity (Yang et al., 2017). Additionally, *in vivo* and artificial gut models have been used to demonstrate that antibiotics induce shifts in the redox potential of the gut, causing changes in respiratory activity, as well as long-term alterations in the composition of the gut microbiome (Reese et al., 2018). These changes reflect what has been found by researchers *in vitro*, linking antibiotics to redox-related changes that complement their own activity to induce lethality (Dwyer et al., 2014, Dwyer et al., 2015; Grant et al., 2012; Jensen et al., 2014; Lobritz et al., 2015).

CLINICAL CONTEXTS OF OXYGEN AVAILABILITY

Both *in vitro* and *in vivo* results indicate that oxygen content is a key determinant of antimicrobial efficacy and the extent of microbiome disruption. Another factor supporting the role of oxygen availability in antibiotic susceptibility is the well-established clinical difficulty of treating anaerobic infections. Anaerobes constitute a significant component of the normal human microflora in body sites with low oxygen, including the gut, oral cavity, and female genital tract but can cause severe infections under certain circumstances (Finegold 1995; Brook 2004). They specifically cause infection upon entering and proliferating within a previously sterile site, usually after disruption of integumentary or mucosal membrane integrity due to trauma, surgery, necrosis, or ischemia (Lee 2009). In general, anaerobes are particularly prevalent on mucous membranes where they cause localized infections as is the case in the oral cavity (Sabiston and Gold 1974; Brook 2016), abdominal cavity (Brook and Frazier 2000; McDonald et al., 2018), and female genital tract (Swenson et al., 1973). However, they can also cause infections in atypical sites such as the central nervous system (Le Moal et al., 2003), middle ear (Brook et al., 1978, 1979), and lower respiratory tract (Brook and Frazier 1993), among others. Anaerobic infections are often polymicrobial in nature and thus present unique clinical challenges in both isolating the causative agent and treatment, which is exacerbated by slow fastidious growth and the potential presence of resistant organisms (Goldstein 1999; Evans et al., 2001; Brook 2004, 2016; Blot et al., 2012).

Several global studies report that the incidence rate of anaerobic infections is similar to the incidence rate of aerobic infections (Swenson et al., 1973; Prasad et al., 2006; Park et al., 2009), with an associated crude

mortality rate of around 25-30% (Wilson and Limaye 2004). Given both high abundance and mortality rates, it is overtly clear that both invasive and opportunistic anaerobes pose a significant threat to human health, and that novel treatment methods and diagnostic tools are greatly needed to combat the growing danger presented by these microbes. Anaerobic bacteria are a major component of the human microbiome, outnumbering aerobes 10:1 or 1000:1 depending on the anatomical site where they are located (Finegold 1995; Nagy 2010). Many different species of anaerobes, particularly those residing on mucosal surfaces, can play a role in human infections (Jousimies-Somer 2002; Horz et al., 2005). For example, species from the Fusobacteria, Bacteroidetes, Actinobacteria, and Firmicutes phyla that are found in the oral cavity have been implicated in tooth and periodontal infections (Newman 1984; Garrett and Onderdonk 2010; Oh et al., 2015), while bacterial vaginosis has been linked to Bacteroidetes and Actinobacteria species (Taylor et al., 1982; Eschenbach 1993; Africa et al., 2014; Lewis et al., 2016). Intra-abdominal infections (IAI) can be caused by a wide variety of opportunistic pathogens that bloom during gut dysbiosis, and examples of such microbes include *C. difficile* (Leffler and Lamont 2015; Czepliel et al., 2019) and species in the Proteobacteria phylum, most notably members of the *Enterobacteriaceae* family (de Ruiter et al., 2009; Blot et al., 2012; Bäumlner and Sperandio 2016). The pathogenic potential of a variety of commensal anaerobes, their tendency to form polymicrobial infections, and their ability to infect nearly any anatomical location complicates treatment methods.

Treatment of anaerobic infections is typically dictated by the patient's clinical presentation, the site of infection, and the presence or absence of abscesses (Blot et al., 2012; Noor and Khetarpal 2019). In general, treatments attempt to limit the local and systemic spread of the infection, and this can be accomplished in a magnitude of ways (Brook 2016). For example, antitoxins can be used to neutralize specific toxins associated with anaerobic infections such as the botulinum and tetanus toxins (Hassel 2013). Surgical interventions allow physicians to alter the microbial growth environment by draining pus, improving circulation, and debriding necrotic tissue (Brook 2016; Mazuski et al., 2017). Surgery is an important form of intervention because anaerobic infections will often persist if abscesses are not drained (Anderson et al., 1976; Cramer et al., 2016; Noor and Khetarpal 2019). Antimicrobial drugs are often necessary, but their efficacy in an anaerobic environment can be limited, as discussed above. Some of the most commonly prescribed antibiotics that can clear anaerobic infections include metronidazole, clindamycin, the carbapenems, and several combinations of a beta-lactamase inhibitor and a beta-lactam (Brook 2007; Noor and Khetarpal 2019). Drug choice depends on infection site, patient risk factors, and the types of bacteria that are likely to be present based on empiric evidence. Since species identification is not routinely performed for anaerobic infections, broad-spectrum antibiotics and combination drug therapy are usually prescribed to combat the different types of bacteria that can be present (Brook 2016). Rising resistance rates to all of these antibiotics necessitates the need for better targeted treatments and an assessment of the local oxygen content may provide an additional factor that drives targeted therapy.

CONCLUSIONS AND POTENTIAL DIRECTIONS

We propose that the location of bacteria in the gut and subsequent environmental pressures, most notably oxygen availability, lead to variation in the response of a microbial community to antibiotic treatment. Several studies have identified key changes in the microbial composition along the longitudinal axis of the gastrointestinal tract after antibiotic administration (Croswell et al., 2009; Buffie et al., 2012; Lee et al., 2020). However, these studies are limited in scope and do not capture the full story of biogeography-based antibiotic susceptibility in the gut. The unique role of gut biogeography and microflora on antibiotic susceptibility is not well understood despite how essential these factors are for predicting and alleviating the potential negative off-target effects of antibiotic therapies.

Although the adverse effects of long-term antibiotic exposure are well documented (Guarner and Malage-lada 2003; Marchesi et al., 2007; Hsiao et al., 2013; Stefka et al., 2014; Lewis et al., 2015; Maurice et al., 2014; Blaser 2011; Chang et al., 2008), few studies have investigated the immediate impacts of antibiotics on the human gut microbiome. This is important because antibiotic-induced changes can generally be separated into two time frames: immediate drug-induced impacts and the long-term perturbations caused by disruptions in microbial and metabolic networks. A few studies have recently shown that short-term exposure to antibiotics induces dramatic changes to the microbiota within 12 hours (Cabral et al., 2019, Cabral et al., 2020), but additional work is needed to fully elucidate the immediate impact on microbial composition. Defining these rapid onset changes is key for determining the impact of location and metabolic environment on differential antibiotic susceptibility in the gut. Unfortunately, studies looking at short-term

differential susceptibility in different parts of the gut are currently lacking, impacting our capacity to make robust predictions about the efficacy of antibiotics in divergent metabolic environments.

In characterizing the factors that drive differential drug susceptibility within the gut, it is critical to first establish the environmental pressures that resident taxa encounter, including but not limited to oxygen content, density of the epithelial mucosa, bacterial load, and relative concentrations of host- and microbiota-accessible carbon sources (Figure 1A). The gastrointestinal tract is a dynamic ecosystem, and these environmental gradients will almost certainly modify the metabolic activity of resident taxa, thus altering antibiotic susceptibility. As such, we must be cognizant of these factors when making predictions about drug efficacy in a given region of the gut microbiota.

In addition to environmental control, the polymicrobial composition of a given intestinal section will also impact the metabolic capacity of the resident taxa. Variations in prominent species will change microbial cross-feeding networks and available metabolite pools as different combinations of microbes effectively compete for microbiota-accessible nutrient sources. For example, mucin degradation products and short-chain fatty acids released by *Akkermansia muciniphila* are utilized by Ruminococcaceae and Lachnospiraceae members, which in turn produce butyrate and vitamin B₁₂, driving a syntrophic network (Figure 1B) (Belzer et al., 2017). When considering the combined impact of environment and taxonomic composition, we can begin to predict potential gradients of drug susceptibility within the gastrointestinal tract (Figure 1C). In the case of the commonly prescribed broad-spectrum drug class beta-lactams, we can propose that short-term susceptibility will be markedly higher for Proteobacteria and Firmicutes compared to Bacteroidetes and Actinobacteria (Cabral et al., 2019). This is because beta-lactams are more effective against aerobic, gram-positive bacteria compared to anaerobic, gram-negative bacteria. However, those Firmicutes that reside in the colon employ anaerobic metabolism, potentially resulting in decreased susceptibility. It is important to note that susceptibility patterns will fluctuate greatly based on the type of antibiotic considered and that individual species do not always follow phylum trends. Future work should consider these environmental conditions when identifying differential responses to beta-lactams, and other antibiotic classes, in the human gut.

Going forward, there are several avenues to explore when it comes to qualifying how the microbiome will respond to antibiotic treatment in different portions of the gut. Further characterizing the immediate impacts of short-term antibiotic exposure in different areas of the gut could help to identify which taxa within the microbiome survive and why, ultimately giving a deeper understanding of the subsequent dysbiosis. Beyond this, understanding host-mediated alterations in microbial metabolism, such as those driven by dietary modification, will further enhance our understanding of antibiotic-induced dysbiosis. Recently, Cabral et al. demonstrated that consumption of a high-sugar/high-fat “Western”-style diet caused increased susceptibility to ciprofloxacin within the murine cecal microbiota (Cabral et al., 2020). This work highlights the need for an expanded examination of dietary intake and antibiotic activity specifically regarding drug class, dietary composition, and induced-metabolic changes to multiple regions of the gut microbiome. A promising avenue of study would be a similar evaluation of fiber intake and antibiotic activity, as preliminary evidence suggests that prioritization of polysaccharide fermentation by the gut microbiota can be protective in certain taxa (Cabral et al., 2019; Desai et al., 2016). Another area that merits exploration is that of gut oxidation state. Given that antibiotic lethality can come, in part, from the generation of ROS, understanding the factors that dictate colonic oxidation is key in accurately predicting drug activity *in vivo*. This could be examined by the introduction of a range of antioxidant compounds in the host diet, paired with subsequent evaluation of antibiotic-induced damage. A final area of importance is intestinal inflammation. Localized inflammation in the intestinal tract can induce short-term increases in colonic oxygenation, thus changing the aerobic environment, and most likely directly modifying the taxonomic composition of the gut and its functional response to antibiotic challenge. Ultimately, studying how these conditions affect the susceptibility of the microbiome to antibiotics is essential for creating accurate predictions of *in vivo* drug activity as well as developing informed clinical practice that may mitigate antibiotic-induced dysbiosis.

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AUTHOR CONTRIBUTIONS

L.R.H. and A.J. contributed equally to this work. L.R.H. and A.J. performed the literature review and wrote the manuscript. L.R.H., A.J., J.I.W., and R.N. created the figure. J.I.W., R.N., S.P., and P.B. prepared the manuscript for publication. P.B. conceptualized the work. All authors have reviewed and approved this manuscript's final version.

DECLARATION OF INTEREST

The authors declare no competing interests.

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