SECONDARY EFFECTS OF ANTIBIOTIC EXPOSURE

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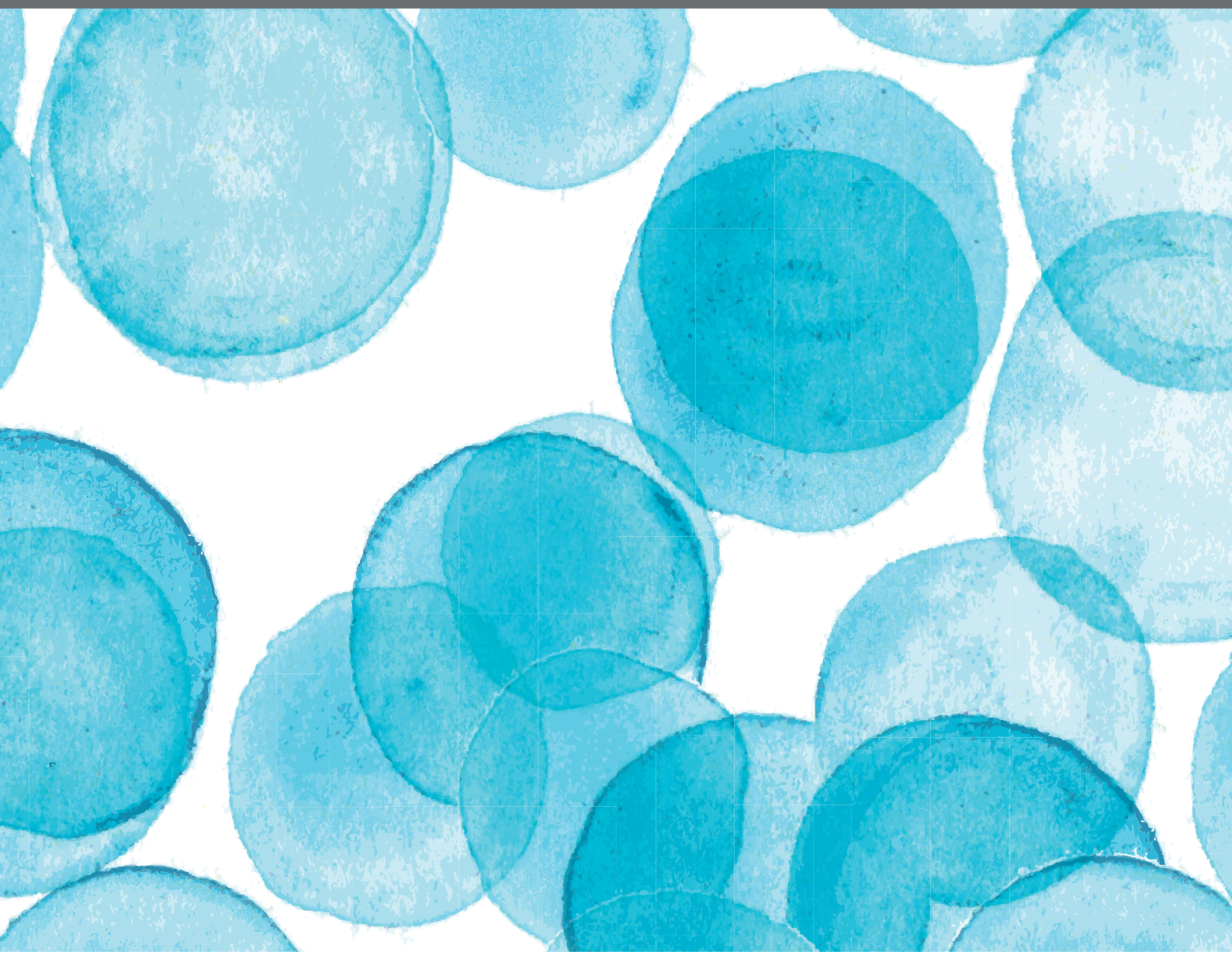

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SECONDARY EFFECTS OF ANTIBIOTIC EXPOSURE

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EDITORIAL
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Editorial: Secondary Effects of Antibiotic Exposure

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Editorial on the Research Topic

Secondary Effects of Antibiotic Exposure

Most commonly used antibiotics have origins in the chemical wars between diverse microbes competing over millennia in crowded environmental niches, like soil. This enduring need to attack their neighbours has made some microbes ruthless killers armed with the ultimate weapon—antibiotics. Antibiotics target biological functions central to the growth or survival of bacteria with an exquisite specificity that has been honed through countless selective iterations over a very long time.

However, their touted, known mechanisms of action may not be so simple.

The way cells respond to antibiotics are complex and multifactorial, both for the bacterial pathogens and infected hosts. For example, bacteria treated with antibiotics can display a plethora of alterations—physiological, behavioural or regulatory—that are seemingly unrelated to the direct killing mechanisms of the antibiotic. These "off target" secondary impacts of antibiotics can be difficult to explain.

Some secondary impacts might reflect alternative biological functions of the molecules, related to the needs of native producing organisms, such as chemical signalling. Alternatively, they may be collateral effects that do not alter the fitness of the antibiotic producers, or poorly defined downstream consequences of the primary antibiotic targeting mechanism. These illicit effects occur for both fully and semi-synthetic antimicrobials.

This Research Topic in Frontiers in Microbiology focuses on these underexplored secondary effects of antibiotics which impact the physiology, behaviour, and evolution of bacterial, host and other co-localised cells. The secondary effects are frequently subtle, lurking beneath the surface of the primary killing/inhibition action, but become apparent through anecdotal observations, or after prolonged antibiotic use. These effects reveal the secret double lives of antibiotics that have sidestepped significant attention, but whose impacts extend to alterations in bacterial virulence, resistance development and treatment efficacy. The papers in this Research Topic describe targeted and cutting-edge investigations into antibiotic secondary effects and their mechanistic bases. The Research Topic comprises of seven papers, including three reviews and four original research articles that discuss the antibiotic secondary effects that are evident throughout all

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walks of life. The imperative review papers in the collection discuss the secondary effects of antibiotics on microbial biofilms (Penesyan et al.), antibiotic-induced mutagenesis, as measured using microscopy (Revitt-Mills and Robinson) and bacterial tolerance of antibiotics (Sulaiman and Lam). The outstanding research papers showcase the broad cellular and regulatory effects that the last-line antibiotic tigecycline has beyond inhibiting translation (Li et al.), how antibiotics can trigger increased gene transfer *via* quorum sensing (Shu et al.,), the complex interactions between antimalarials and antibiotics in bacteria (Olateju et al.), and the impact that antibiotics have on animals with brain ischemia (Lee et al.).

Antibiotics impose huge selective pressures on bacteria, and their widespread use is a major anthropogenic driver of bacterial evolution. Two reviews in the collection describe processes related to bacterial adaptation to antibiotics. Revitt-Mills and Robinson address induced-antibiotic mutagenesis, whereby some antibiotics can cause higher than normal mutation rates in bacterial populations, e.g., by modulating processes of DNA synthesis and repair, which may promote population heterogeneity. This heterogeneity theoretically poses greater potential for mutational resistance selection, but the importance of induced-antibiotic mutagenesis in the development of antibiotic resistance has been technically very hard to study because of the range of cellular processes occurring concurrently that could influence resistance development. Revitt-Mills and Robinson describe the use of microscopy to observe directly the molecular mechanisms involved in induced-antibiotic mutagenesis that may then be linked to resistance outcomes.

In the second review describing bacterial adaptation to antibiotics, Sulaiman and Lam explore the phenomenon of antibiotic tolerance, in which a sub-population of bacterial cells can persist during antibiotic exposure in a dormant state. Upon removal of antibiotic selection, these bacterial persister cells may reseed a new bacterial population. Persister cells are not genetically different to their non-persister counterparts. However, various mutations may lead to a higher likelihood of persister cell formation. Sulaiman and Lam catalogue and describe these mutations as identified in laboratory evolution studies.

Bacteria are often found within biofilms, which are surface-associated structures where bacteria, and other microbes, are embedded within a self-produced protective matrix. When bacteria are in biofilms, they are more resistant to many antibiotics as compared to their planktonic counterparts. This results in significant challenges in treating biofilm-associated infections. Given this, many studies have focused on the mechanisms underpinning this increased resistance. In this special edition a very timely review by Penesyan et al., explores how bacterial cells in biofilms respond to sub-inhibitory concentrations of antibiotics and how this impacts upon treatment in healthcare and industrial settings.

Insights into the secondary effects of antibiotics can be gleaned through unbiased genome scale analyses, such as transcriptomics. Tigecycline is an important antibiotic in our arsenal, that is saved for the most difficult and deadly infections, like those caused by the notoriously resistant pathogen *Acinetobacter baumannii*. Its mechanism of action is *via* protein translational inhibition, yet (Li et al.) used transcriptomic approaches to reveal a global network of genes for tigecycline resistance, many of which were out-of-scope for the expected if this antibiotic simply inhibits translation, including changes in toxin-antitoxin systems, peptidoglycan biosynthesis and unrelated antibiotic resistance gene.

Vancomycin is used as the last line of defence against serious infections caused by a range of Gram-positive bacteria. Vancomycin resistant enterococci are widespread and on the WHO priority list of bacteria for which new antibiotics are urgently needed. Pheromone-induced conjugation facilitates rapid dissemination of antibiotic resistance in enterococci populations. Shu et al., demonstrate that transfer of vancomycin resistance in *Enterococcus faecalis* increased dramatically when cells were treated with other antibiotics, namely streptomycin and spectinomycin. The finding that treatment with commonly used antibiotics leads to dissemination of plasmids encoding resistance to different antibiotics is a terrifying and problematic example of the knock-on effects of antibiotic usage.

Microbial co-infections occur relatively frequently, particularly in situations where one pathogen is immunosuppressive, resulting in the need to co-administer different classes of antimicrobials. This raises a very interesting but complicated phenomenon, where the primary or secondary impacts of one antimicrobial may modulate the activity of others. Olateju et al. performed a range of assays to investigate this occurrence between quinoline antimalarials and the beta-lactam antibiotic ampicillin. This research revealed that chloroquine and quinine have limited antibacterial activity alone, but the combination of one of these compound and ampicillin results in at least additive bacterial inhibitory effects. This could be important in the design of treatments for malarial/bacterial co-infections.

Antibiotics are recommended to prevent pathogen infection in patients with brain injury, however in addition to reducing infection risk (Lee et al.) report that the oral administration of antibiotics may lead to further cognitive impairment in patients with brain ischemia. They demonstrate that oral administration of vancomycin and ampicillin to mice with brain ischemia, may lead to neuroinflammation, *via* the increased translocation of LPS into the brain caused by changes in their gut microbiome.

Overall, these reported effects likely represent only the tip of the iceberg of the known secondary roles that antibiotics play within cellular environments. Antibiotics clearly lead a double life that is only now being revealed; in addition to being ruthless and effective killers of bacteria, antibiotics have multifaceted roles in nature that are often overlooked. Future research, especially those employing global molecular approaches, will allow us to fully comprehend the overall consequences of antibiotic treatment on both bacterial pathogens

and host cells. Only then can we begin to understand and comprehend the full cellular effects of these complex killing molecules.

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Secondary Effects of Antibiotics on Microbial Biofilms

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Biofilms are assemblages of microorganisms attached to each other, or to a surface, and encased in a protective, self-produced matrix. Such associations are now recognized as the predominant microbial growth mode. The physiology of cells in biofilms differs from that of the planktonic cells on which most research has been conducted. Consequently, there are significant gaps in our knowledge of the biofilm lifestyle. Filling this gap is particularly important, given that biofilm cells may respond differently to antibiotics than do planktonic cells of the same species. Understanding the effects of antibiotics on biofilms is of paramount importance for clinical practice due to the increased levels of antibiotic resistance and resistance dissemination in biofilms. From a wider environmental perspective antibiotic exposure can alter the ecology of biofilms in nature, and hence disrupt ecosystems. Biofilm cells display increased resilience toward antibiotics. This resilience is often explained by mechanisms and traits such as decreased antibiotic penetration, metabolically inactive persister cells, and intrinsic resistance by members of the biofilm community. Together, these factors suggest that cells in biofilms are often exposed to subinhibitory concentrations of antimicrobial agents. Here we discuss how cells in biofilms are affected by the presence of antibiotics at subinhibitory concentrations, and the possible ramifications of such secondary effects for healthcare and the environment.

Keywords: antimicrobials, subinhibitory, antibiotic resistance, infection, environmental pollution

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INTRODUCTION

Biofilms are usually defined as microbial assemblages that can be either surface-associated, or present as an unattached but aggregated cell biomass. Biofilm cells are encased within a self-produced matrix comprised of extracellular polymeric substances (EPS) including polysaccharides, extracellular DNA (eDNA), proteins and lipids. The EPS matrix provides protection for the cells inside biofilms by acting as a shield, and is central to biofilm structure and integrity (Flemming and Wingender, 2010).

Biofilms are recognized as the predominant form of bacterial and archeal life. While the evaluation of biofilm vs. planktonic lifestyle is inconclusive for oceanic environments

due to common sampling techniques that do not distinguish between the two lifestyles, more accurate estimates suggest that on the surface of the Earth biofilms dominate all habitats and account for ∼80% of bacterial and archeal cells (Flemming and Wuertz, 2019). Biofilms are common both in nature and in association with host organisms. They protect bacteria from external threats including protozoan grazing (Matz et al., 2005) and antibiotic exposure (Penesyan et al., 2015).

Biofilm communities are important for ecosystems. They drive biogeochemical processes and play fundamental roles in nutrient cycling and bioremediation (Yadav, 2017; Flemming and Wuertz, 2019). Biofilms are also an essential part of human, animal and plant microbiomes (de Vos, 2015). However, both in the environment and in association with a host, biofilm formation by bacteria can pose major challenges and risks.

Biofilms are the leading cause of biomass build-up on submerged surfaces (pipes, catchments, filters, ship hulls), becoming reservoirs for infection and also resulting in equipment failure and decreased productivity. Since biofilms are extremely resilient to external stressors, such as antimicrobials, they are difficult to eradicate. Thus, biofilms are estimated to cost billions of dollars to the United States industry alone, in the maintenance and replacement of essential infrastructure such as water transmission and distribution networks, as well as in the maintenance and cleaning of ship hulls (U.S. EPA, 2002; Schultz et al., 2011).

More than 60% of all infections in hospitals and healthcare settings world-wide are due to bacterial pathogens that form biofilms (Moscoso et al., 2009). Due to the additional layer of protection provided by the biofilm lifestyle, biofilm bacteria can display an increase in antibiotic resistance by orders of magnitude compared to their planktonic state, and are therefore a major obstacle to infectious disease treatment (Penesyan et al., 2015). As a result, nearly 80% of biofilm-related infections do not respond to antibiotic treatment (Moscoso et al., 2009) leading to increased healthcare costs and patient mortality/morbidity. Estimates suggest that biofilm infections cost nearly $100 billion annually to the United States healthcare system and cause more than 500,000 deaths in the United States alone (Wolcott et al., 2010).

Many antibiotics derive from natural products found in environmental ecosystems. Bacteria have hence had an opportunity to encounter these compounds during billions of years of evolution. Consequently, microorganisms have adapted by arming themselves with strategies to overcome toxic effects of antibiotics. Moreover, microbes can use antibiotics as cues or signals, often developing physiological responses that provide them with an ecological advantage that increases their survival (Linares et al., 2006).

The ability to control biofilm formation is of great importance in medicine and industry. As a result, the effects of antibiotics on biofilms have largely focused on the recalcitrance of biofilms toward antimicrobials, and the development of tools to reduce that resilience, thus making treatments more effective. In this review we focus on rarely considered but important effects that antimicrobials have on biofilm cells at subinhibitory concentrations. This approach can help to better understand the roles of antimicrobials in biofilm biology, and to recognize possible unintended consequences of our current strategies to control infections and biofilm growth.

BIOFILMS IN INFECTIONS

Antibiotics can have a variety of effects on microbial biofilms. These effects can be complex and depend on many factors, such as the concentration of antibiotics to which the organisms are exposed, specific growth conditions, and the characteristics of the organism itself. The effect of antibiotics on biofilms in infections can be roughly evaluated as two seemingly opposing effects: disrupting existing biofilms or enhancing biofilm formation.

Disrupting Biofilms: A Double-Edged Sword

The biofilm-disruptive effects of some antibiotics can seem promising for antibiotic therapy. Destabilization of the biofilm structure can be regarded as a way to eliminate the additional protection provided by biofilms, thus making the biofilms more penetrable for antimicrobials, and biofilm cells more susceptible to antibiotics. However, this strategy is fraught with peril as biofilm structure destabilization and loosening of the biofilm matrix can lead to cellular detachment and further dissemination of biofilms, potentially leading to increasingly severe and long-term consequences.

In a recent study Díaz-Pascual et al. (2019) examined the effects of antibiotics commonly used to treat cholera on biofilms of *Vibrio cholerae*. Transient exposure to translation-inhibiting antibiotics such as tetracycline caused alterations in cell shape and physiology that resulted in large-scale changes in biofilm architecture and the dismantling of cell-matrix associations. This effect may be considered favorable for *V. cholerae* biofilm eradication, as loosening of biofilm structures may allow antimicrobials better access to the biofilm interior. However, disrupting the biofilm structure may detach cells that can then serve as inocula for new points of infection, and hence cause spreading of the infectious agent.

Clinically relevant concentrations of colistin, a polymyxin antibiotic that primarily targets Gram-negative bacteria via disrupting bacterial membranes, have been found to also destabilize the biofilm matrix of clinical isolates of the Gram-positive pathogen *Staphylococcus aureus* as well as Gram-negative *Escherichia coli* (Klinger-Strobel et al., 2017). The electrostatic interaction of the amphiphilic molecule colistin with matrix exopolysaccharides was suggested as a possible mechanism. Similar effects were also observed when biofilms of the opportunistic pathogen *Pseudomonas aeruginosa* were exposed to subinhibitory concentrations of the cation-chelator antibiotic nitroxoline (Sobke et al., 2012).

Yoshii et al. (2017) have shown that norgestimate, a compound primarily used for oral contraception and hormone replacement therapy (Henzl, 2001) but also investigated as a potential antibiofilm/antibiotic agent, was able to inhibit biofilm formation by staphylococcal strains. It did so via inhibiting production of a polysaccharide adhesin and proteins in the extracellular matrix, thus affecting biofilm structure and integrity (Yoshii et al., 2017).

Likewise, *Enterococcus faecalis*, an opportunistic pathogen that is the most prevalent enterococcal species identified in healthcare-associated infections, displayed changes in biofilm architecture and restructuring of biofilm monolayers into complex three-dimensional biofilms upon the exposure to subinhibitory concentrations of clinically relevant antibiotics. This effect was seen across antibiotics with diverse modes of action, including daptomycin (disrupts bacterial cell membranes) and gentamicin (protein synthesis inhibitor) (Dale et al., 2017). The effect was related to the cellular stress response and was accompanied by increased biofilm detachment. While this response could make these bacteria more susceptible to antibiotics or to immune responses, biofilm detachment could also be advantageous for *E. faecalis* in terms of dissemination and long-term reproduction during infection.

Enhancing Biofilm Formation and Resilience

There is mounting evidence (reviewed in Kaplan, 2011) to suggest that subinhibitory concentrations of many antibiotics are able to enhance biofilm formation by pathogens.

Exposure of *Campylobacter jejuni*, a bacterium often associated with human gastroenteritis, to various antibiotics led to enhanced biofilm formation in antibiotic susceptible strains, suggesting that the response reflects a survival strategy by these strains (Teh et al., 2019). A similar biofilm enhancing effect was observed for *Leptospira* spp. after treatment with subinhibitory concentrations of the antibiotics doxycycline and tetracycline, the drugs of choice for leptospirosis (Vinod Kumar et al., 2018).

The macrolide antibiotic azithromycin (protein synthesis inhibitor) is reported to interfere with quorum-sensing-dependent virulence factor production, biofilm formation, and oxidative stress resistance in *P. aeruginosa*. This organism is a major bacterial pathogen associated with cystic fibrosis chronic infections, and azithromycin treatment led to improved patient outcomes (Equi et al., 2002; Saiman et al., 2003; Nalca et al., 2006). However, while the antibiotic was effective during its first year of administration, it showed a poor efficacy thereafter (Tramper-Stranders et al., 2007; Principi et al., 2015; Willekens et al., 2015). Using *in vitro P. aeruginosa* biofilms, it was demonstrated that azithromycin delayed biofilm formation, but these effects were short-lived and appeared to be specific to the initial stages of biofilm development. After 48 h, an emerging resistant phenotype was able to overcome the inhibitory effect of azithromycin and result in a very robust biofilm (Gillis and Iglewski, 2004). Thus, the limited long-term effect of azithromycin on *in vitro* microbial biofilms may explain its failure in the prolonged treatment of cystic fibrosis infections in clinical practice.

Subinhibitory concentrations of aminoglycoside antibiotics (protein synthesis inhibitors) were found to induce biofilm formation in the opportunistic human pathogens *P. aeruginosa* and *E. coli* (Hoffman et al., 2005). This effect was described as a specific defensive reaction to the presence of antibiotics and was linked to alterations in the level of c-di-GMP, an important bacterial second messenger that regulates cell surface adhesiveness, biofilm formation and virulence (D'Argenio and Miller, 2004; Ryan, 2013). In addition, increased biofilm formation in *E. coli* upon antibiotic exposure has been shown for the exposure to the bacterial cell-wall targeting antibiotics colistin and carbenicillin and for the translation-inhibiting drug tetracycline (Boehm et al., 2009). A biofilm-enhancing effect of tetracycline was also recently demonstrated for the nosocomial pathogen *Acinetobacter baumannii*, as a response to low-level antibiotic exposure, and was accompanied by rapid genome evolution and the generation of antibiotic-resistant mutants (Penesyan et al., 2019).

The close proximity of microbes within biofilm aggregates and the abundance of eDNA may facilitate horizontal gene transfer and the spread of resistance determinants (Penesyan et al., 2015). Exposure to subinhibitory antibiotic concentrations can promote the growth of resistant and/or more fit variants by both selecting for preexisting mutants and by promoting new mutations (Andersson and Hughes, 2014; Ahmed et al., 2018; Santos-Lopez et al., 2019). A recent study demonstrated that such effects can be extraordinarily rapid in biofilms. Within 3 days of exposure, subinhibitory antibiotic concentrations can help generate and select an array of mutations that confer resistant phenotypes (Penesyan et al., 2019).

The biofilm lifestyle and the presence of antibiotics are known to induce the bacterial stress response, including the general stress response, oxidative stress response accompanied by the induction of central carbon metabolites and elevated TCA cycle intermediates, stringent response and the SOS response. The latter increases mutation rates and facilitates horizontal transfer of DNA, including antimicrobial resistance determinants (Kohanski et al., 2010; Gutierrez et al., 2013; Belenky et al., 2015; Strugeon et al., 2016; von Wintersdorff et al., 2016; Gillings, 2017; Liu et al., 2019). Thus, biofilms may constitute specific foci of genetic adaptation and evolution, leading to the selection of subpopulations with a greater ability to withstand current and future antibiotic exposure.

Antibiotic exposure can also lead to transient adaptive physiological changes. *Streptococcus intermedius*, a commensal bacterium associated with periodontitis, fatal purulent infections and brain and liver abscesses, showed increased biofilm formation upon exposure to subinhibitory concentrations of three antibiotics with distinct modes of action and different bacterial targets. These included ampicillin (bacterial cell wall synthesis inhibitor), ciprofloxacin (transcription inhibitor), and tetracycline (translation inhibitor). The increased biofilm formation was linked to the *S. intermedius* AI-2/LuxS quorum sensing system being activated in the presence of antibiotics (Ahmed et al., 2009). In contrast, ciprofloxacin, as well as azithromycin (protein synthesis inhibitor) and ceftazidime (bacterial cell wall inhibitor), were found to decrease the expression of quorum-sensing related genes in *P. aeruginosa* (Skindersoe et al., 2008). These suggest that such effects can be both strain- and antibiotic-specific, and, therefore, hard to predict.

Changes in the morphology of biofilm cells (i.e., rounding, blebbing, and alteration of cell size) were observed in *Klebsiella pneumoniae* biofilms in the presence of sublethal concentrations

of β-lactam carbapenems (cell wall inhibitors) imipenem, meropenem and doripenem (Van Laar et al., 2015). In general, despite morphological differences observed, there were no significant differences in viability in most treated biofilms, with the exception of a slight decrease in viability observed in doripenem-treated biofilm cells. Cells in biofilms treated with all three antibiotics returned to their normal pre-treatment morphology soon after the removal of the antibiotic and showed no significant change in viability. A number of genes were implicated in these morphological transitions, including genes involved in peptidoglycan biosynthesis and catabolism. Genes involved in the general stress response, virulence and antibiotic resistance were differentially expressed in the presence of imipenem, causing profound changes in cellular physiology. Upregulation of genes involved in the formation of antibiotic tolerant persister cells was also observed in imipenem-treated biofilms, possibly contributing to the ability of these biofilms to remain viable even with a carbapenem treatment of 1,000× the MIC (Minimum Inhibitory Concentration, as tested in planktonic cultures) (Van Laar et al., 2015).

Exposure to the β-lactam antibiotic imipenem induced genes coding for alginate biosynthesis in *P. aeruginosa* and led to increased biofilm volume (Bagge et al., 2004). Alginate is an important polysaccharide component of *P. aeruginosa* biofilms that serves to protect bacteria from adverse conditions while also enhancing its adhesion to surfaces (Boyd and Chakrabarty, 1995). Alginate production by *P. aeruginosa* underlies its conversion to mucoid phenotype and is linked to the development of impaired lung function in cystic fibrosis patients (Li et al., 2005). The increased expression of alginate in response to imipenem may be an unintended adverse consequence of imipenem treatment of *P. aeruginosa* infections in cystic fibrosis (Bagge et al., 2004).

Beta-lactam antibiotics methicillin, ampicillin, amoxicillin, and cloxacillin were shown to induce biofilm formation in *Staphylococcus aureus* strains, including methicillin-resistant *S. aureus* (MRSA). This effect was linked to autolysin-dependent release of eDNA, an important constituent of biofilms (Kaplan et al., 2012). Likewise, Yu et al. (2018) demonstrated that subinhibitory concentrations of bacterial cell-wall targeting antibiotics led to enhanced biofilm formation and increased density of biofilm cells in the prominent nosocomial pathogen *E. faecalis*. This effect was associated with increased cell lysis accompanied by a surge in eDNA levels, suggesting that such effects may take place in a variety of Gram-positive pathogens.

Bacillus subtilis was shown to form biofilms in response to a variety of structurally dissimilar bioactive natural products of microbial origin, including those produced by *B. subtilis* itself. It was suggested that these molecules cause potassium leakage across the cytoplasmic membrane. This results in the activation of a protein kinase that sets in motion a chain of regulatory events, inducing the expression of genes involved in the synthesis of EPS matrix constituents – a central component of the biofilm architecture (López et al., 2009). A similar biofilm-enhancing effect in *B. subtilis* was demonstrated for a variety of thiopeptide antibiotics known to interfere with ribosomal functioning. This suggests that, besides their anthropogenic application for inhibiting bacterial growth, antibiotics may have complex roles as signaling molecules that specifically modulate bacterial phenotypes (Bleich et al., 2015; Townsley and Shank, 2017).

The notion that antibiotics have intrinsic environmental roles and can act as signals in diverse biological processes is not novel (Yim et al., 2007). Linares et al. (2006) demonstrated a signaling role for antibiotics by showing the biofilm enhancing effects of several antibiotics on the important opportunistic pathogen *P. aeruginosa*, accompanied by changes in physiology and ecological behavior. Thus, in such scenarios, at subinhibitory levels the antibiotics become beneficial to the pathogen, promoting its survival, hence having the opposite effect to their intended use as growth inhibitors.

The impacts of subinhibitory concentrations of antibiotics on a range of phenotypic outcomes, including enhanced survival, complicate biofilm eradiction efforts. Rather than achieving biofilm removal, antibiotics may also strengthen and enhance the survival of microbes by increasing the protection provided by biofilms.

BIOFILMS IN NATURE

There is a continuous discharge of effluents from human activities, including industrial and hospital waste, into water reservoirs such as rivers and lakes. Along with wastewater, antibiotics used in clinical practice and agriculture often end up in environmental reservoirs and in coastal marine environments (Nödler et al., 2014; Nakayama et al., 2017). Here, they add to the burden of recalcitrant anthropogenic pollutants known as Trace Organic Compounds (TOrCs) (Basiuk et al., 2017). Microorganisms inhabiting polluted water bodies are consequently exposed to varying low concentrations of chemical pollutants, including antibiotics (Yadav, 2017; Chow et al., 2021).

Biofilms associated with infections in the human body tend to involve a limited number of species (Burmølle et al., 2010). In contrast, environmental biofilms are often diverse communities that include multiple taxonomic groups with a multitude of functions. Compared to the wealth of research data on antibiotics and their effects on biofilms in clinical settings, data on environmental biofilms are very limited.

As discussed above, antibiotics have diverse effects on various biofilm communities, including enhanced biofilm formation, increased resistance and the spread of antimicrobial resistant phenotypes and genotypes. The increasing presence of antibiotic compounds in the environment is therefore of great concern. Environmental biofilms downstream of a sewer overflow site exhibit increased antibiotic resistance (Kaeseberg et al., 2018). Moreover, antibiotic resistance genes and resistant organisms are often found in natural waterways and can also persist for prolonged periods of time within sediments (Calero-Cáceres et al., 2017; Zhu et al., 2017; Gillings, 2018; Guo et al., 2018; Lépesová et al., 2018). Thus, these environments can serve as reservoirs for antibiotic resistant organisms and further facilitate dissemination of resistance genes.

Triclosan is a bactericidal compound that has been widely used in cosmetics and household cleaners. It is able to enter fluvial

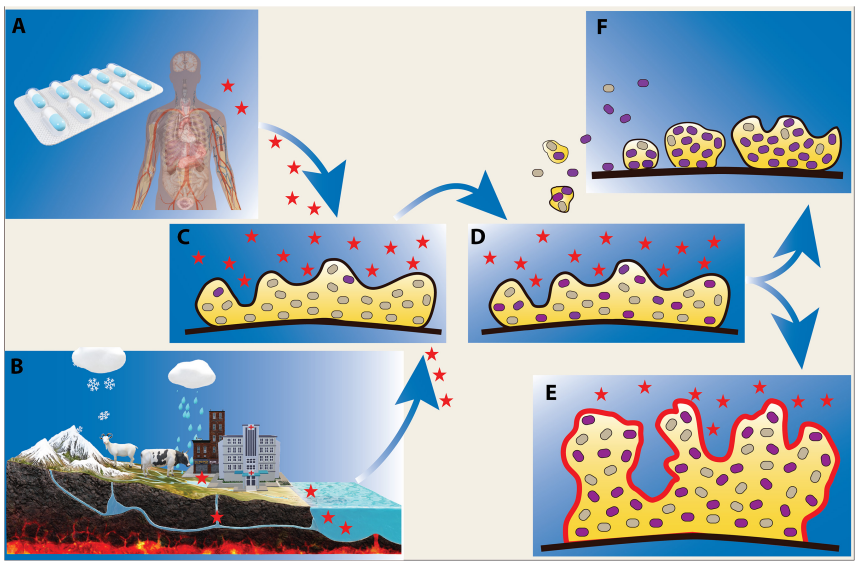

FIGURE 1 | Effect of low levels of antibiotics on microbial biofilms. Biofilm bacteria are often exposed to low levels of antibiotics during infection treatment and antibiotic therapy **(A)**, and in natural water reservoirs where there is a flux of antibiotics from sources such as agriculture, aquaculture, hospital and domestic waste **(B)**. Exposure of biofilms to antibiotics (indicated as red stars) **(C)** can lead to the emergence of antibiotic resistant phenotypes/genotypes **(D)** (shown as purple cells) and facilitate formation of more robust biofilms **(E)**. Upon antibiotic exposure, resistant biofilm bacteria can further disseminate and serve as inocula for potentially more resistant biofilm communities **(F)**.

ecosystems after surviving the degradation steps in wastewater treatment plants (Ricart et al., 2010). Short-term effects of triclosan on fluvial biofilms caused bacterial mortality and the inhibition of photosynthetic activity in autotrophs (Ricart et al., 2010). Another study, however, showed that despite initial widespread bacterial mortality, laboratory-grown multispecies stream biofilms recovered their normal structure and function within a few days or weeks after exposure, highlighting the capacity of biofilms to tolerate and persist in the face of periodic inputs of toxic compounds (Proia et al., 2011).

In another study, Proia et al. (2013) investigated the effects of antibiotics on biofilm communities in the Llobregat River (Northeast Spain) where the antibiotics present as pollutants at the highest concentrations were sulfonamides, followed by quinolones and macrolides. An enhanced bacterial mortality, an increased abundance of *Actinobacteria* and a general decrease of enzymatic activity in biofilms exposed to the polluted water were documented. This indicates that the release of antibiotics into running waters can cause significant structural and functional changes in microbial biofilm communities.

As environmental biofilm communities serve an essential role in ecosystem health and functioning, antibiotic driven disturbances of these communities may have unexpected consequences. In biofilm communities involved in anaerobic ammonium oxidation (annamox), a globally important microbial process that is used in wastewater treatment, the presence of trace amounts of norfloxacin significantly suppresses annamox activity (Zhang X. et al., 2019).

Biofilms themselves can also affect the fate of antibiotics and their impacts in the environment. For example, a recent study has shown that sorption of the fluoroquinolone antibiotic ofloxacin to sediments was inhibited when sediments were coated by biofilms, increasing the concentration of the antibiotics in the aqueous phase and further enhancing the ecological risks associated with the presence of the antibiotic in water reservoirs (Zhang L. et al., 2019).

CONCLUSIONS AND FUTURE PERSPECTIVES

Studying the potential consequences of antibiotic discharge into the environment is of paramount importance for evaluating short-term and long-term effects of antibiotic use. The sources of such antibiotic pollution are diverse, and include hospitals, industry, agriculture, aquaculture and domestic use (**Figure 1**).

The primary application of antibiotics has been at growth-inhibitory concentrations, and research aimed at disclosing the mode of action and outcomes provided by antibiotics has generally been conducted at such concentrations. However, data show that depending on concentration, antibiotic exposure can trigger different physiological responses. Subinhibitory concentrations, in particular, can enhance the growth and resilience of biofilms and promote the dissemination of cells from existing biofilms. Therefore, measures need to be taken to limit unintentional exposure of microbial biofilms to low levels of antibiotics, both in healthcare and environmental settings.

The excessive and often unnecessary use of antibiotics has become a worldwide problem for healthcare as it drives antibiotic resistance in pathogens and hinders our ability to treat infections. Combatting infectious diseases is especially problematic in

developing countries with limited or inadequate access to medications. This problem is exacerbated by the potential use of counterfeit and substandard antibiotic preparations. The most common types of substandard/counterfeit antimicrobial drugs have a reduced amount of the active drug (Kelesidis and Falagas, 2015). The use of such preparations leads to the exposure of pathogens in biofilms to sublethal doses of antimicrobials with potentially far reaching consequences. Better controls of antibiotic usage and substandard/counterfeit antibiotic preparation may help to ameliorate some of the problems discussed above.

The use of proper antibiotic waste management procedures, as well as strategies to reduce the presence of antibiotics in environmental reservoirs using managing aquifer recharge (MAR) systems (Drewes, 2009) and various other biotic (e.g., via microbial degradation) and abiotic (e.g., via sorption, hydrolysis, photolysis etc.) remediation procedures (Kumar et al., 2019) are also necessary to limit the exposure of microbial biofilms to antibiotics and minimize their subsequent secondary effects. To this end much additional work is required to understand the environmental fate as well as biodegradation processes of antibiotics, in order to reduce contaminant loads in the environment.

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All authors have made a direct intellectual contribution to the work and approved it for publication.

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REVIEW
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Antibiotic-Induced Mutagenesis: Under the Microscope

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The development of antibiotic resistance poses an increasing threat to global health. Understanding how resistance develops in bacteria is critical for the advancement of new strategies to combat antibiotic resistance. In the 1980s, it was discovered that certain antibiotics induce elevated rates of mutation in bacteria. From this, an "increased evolvability" hypothesis was proposed: antibiotic-induced mutagenesis increases the genetic diversity of bacterial populations, thereby increasing the rate at which bacteria develop antibiotic resistance. However, antibiotic-induced mutagenesis is one of multiple competing factors that act on bacterial populations exposed to antibiotics. Its relative importance in shaping evolutionary outcomes, including the development of antibiotic resistance, is likely to depend strongly on the conditions. Presently, there is no quantitative model that describes the relative contribution of antibiotic-induced mutagenesis to bacterial evolution. A far more complete understanding could be reached if we had access to technology that enabled us to study antibiotic-induced mutagenesis at the molecular-, cellular-, and population-levels simultaneously. Direct observations would, in principle, allow us to directly link molecular-level events with outcomes in individual cells and cell populations. In this review, we highlight microscopy studies which have allowed various aspects of antibiotic-induced mutagenesis to be directly visualized in individual cells for the first time. These studies have revealed new links between error-prone DNA polymerases and recombinational DNA repair, evidence of spatial regulation occurring during the SOS response, and enabled real-time readouts of mismatch and mutation rates. Further, we summarize the recent discovery of stochastic population fluctuations in cultures exposed to sub-inhibitory concentrations of bactericidal antibiotics and discuss the implications of this finding for the study of antibiotic-induced mutagenesis. The studies featured here demonstrate the potential of microscopy to provide direct observation of phenomena relevant to evolution under antibiotic-induced mutagenesis.

Keywords: stress response, DNA damage, imaging, experimental evolution, recombination

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ANTIBIOTIC-INDUCED MUTAGENESIS: FROM MOLECULES TO CELLS TO POPULATIONS

Mutation is one of the two major mechanisms for the development of antibiotic resistance in bacteria (Woodford and Ellington, 2007). In the laboratory, high-level resistance against most antibiotics can be acquired by bacteria through the development of mutations (Woodford and Ellington, 2007). In clinically relevant bacterial pathogens, resistance to most antibiotics is

acquired through lateral gene transfer; however, mutational resistance dominates in some circumstances. Resistance against fluoroquinolones (Appelbaum and Hunter, 2000; Ruiz, 2003), rifampicin (Gillespie, 2002), oxazolidinones (Long and Vester, 2012), fusidic acid (Nagaev et al., 2001; Besier et al., 2005), and streptomycin (Gillespie, 2002) frequently arises through mutation (Woodford and Ellington, 2007). Commonly these are "target-gene mutations," in which the systems that are targeted by the antibiotic become altered in such a way that the antibiotic no longer binds to its target or is otherwise made ineffective. Another important class of mutations that occur commonly among clinical isolates is loss-of-function mutations in regulators that can lead to increased activity of associated multi-drug efflux pumps (Woodford and Ellington, 2007). Interestingly, the causative agent of tuberculosis, *Mycobacterium tuberculosis*, appears to acquire antibiotic resistance exclusively through mutation, as opposed to lateral gene transfer (Gillespie, 2002).

In many cases, we have a strong understanding of how particular mutations lead to an increase in resistance against a particular antibiotic (Woodford and Ellington, 2007). Much is known about the molecular mechanisms that generate mutations and the evolutionary dynamics that allow them to fix within bacterial populations (Andersson and Hughes, 2017). In the simplest scenario, mutations occur spontaneously, arising from errors made during DNA synthesis (Martinez and Baquero, 2000). Mutants are present in the population prior to antibiotic exposure and are strongly selected for once the population is exposed (Martinez and Baquero, 2000). This spontaneous mutation model serves as the null hypothesis in any study seeking to uncover the origins of a particular antibiotic resistance mutation. It is known, however, that some antibiotics can induce higher mutation rates in bacteria (Mao et al., 1997; Oliver et al., 2000; Blazquez et al., 2018). The role of induced mutagenesis in the evolution of antibiotic resistance remains a lively field of research (Blazquez et al., 2018).

Exposure to antibiotics can elevate mutation rates in bacteria in two ways. Both mechanisms have demonstrated capacity to drive the development of antibiotic resistance in laboratory models (Cirz et al., 2005; Pribis et al., 2019). First, antibiotic exposure often leads to the selection of hypermutator phenotypes; cells that have developed constitutively high mutation rates (Mao et al., 1997; Oliver et al., 2000; Shaver et al., 2002; Blazquez, 2003; Durao et al., 2018). These usually arise from loss-of-function mutations in systems that normally help to maintain low mutation rates, such as mismatch repair enzymes or proofreading exonucleases (Miller et al., 1999; Rodriguez-Rojas et al., 2013; Durao et al., 2018). Second, exposure to antibiotics can induce elevated mutation rates directly, by promoting the activities of error-prone DNA repair systems or by temporarily down-regulating mismatch repair (Frisch et al., 2010; Shee et al., 2011a). It is this second class of phenomena, termed antibiotic-induced mutagenesis or transient hypermutation, that is the primary focus of this review.

Antibiotic-induced mutagenesis is most often linked to the SOS response; a transcriptional program that upregulates a set of genes (~40 in *Escherichia coli*), most of which have functions relevant to DNA damage (Blazquez et al., 2018; Maslowska et al., 2019). Included in the SOS regulon are genes that encode error-prone DNA polymerases (Goodman and Woodgate, 2013; Joseph and Badrinarayanan, 2020). During the SOS response, error-prone polymerases increase the rate at which mutations appear in cells (Kuban et al., 2004; Hastings et al., 2010; Blazquez et al., 2018). In this way, antibiotic exposure begets mutagenesis. The observation of antibiotic-induced mutagenesis led to the proposal of an "increased genetic diversity" hypothesis in the early 2000s: antibiotic-induced mutagenesis drives an increase in the genetic diversity of bacterial populations and thus speeds the development of mutational antibiotic resistance (Radman, 1999; Martinez and Baquero, 2000; Rosenberg, 2001; Cirz et al., 2005). Twenty years later, this idea remains an active field of study (Blazquez et al., 2018).

There is ample evidence that antibiotic-induced mutagenesis occurs. Much of this evidence has been reviewed in detail recently (Blazquez et al., 2018). The primary question facing the field is: does it matter? Does the potential increase in mutation supply brought on by antibiotic-induced mutagenesis ever outweigh the loss of diversity brought on by the antibiotic killing the cells? To our knowledge, only one experimental evolution study has specifically investigated whether antibiotic-induced mutagenesis can play a dominant role in the evolution of antibiotic resistance. This study, which monitored the *de novo* appearance of ciprofloxacin-resistance mutations in a mouse infection model strongly suggested that resistance was dependent on antibiotic-induced mutagenesis (Cirz et al., 2005). As detailed below, assessing the role of antibiotic-induced mutagenesis in evolution requires that the effects of an antibiotic on mutagenesis are experimentally isolated from its effects on cell survival. Currently, this is almost impossible to do in animal models and this ultimately limits the use of top-down approaches toward studying evolution under antibiotic-induced mutagenesis. At the other end of the spectrum, observations made at the level of individual cells and small populations of cells may provide sufficient insight to enable accurate computer modeling of events that are too complex to monitor directly. With enough data, collected under carefully controlled conditions, this offers a potential means to approach the antibiotic-induced mutagenesis problem from the bottom-up.

Antibiotic-induced mutagenesis will only influence evolutionary outcomes in situations where the bacterial cells remain alive long enough to produce new mutations. Thus, it is likely that it occurs infrequently under high antibiotic concentrations, where most cells die quickly. For this reason, antibiotic-induced mutagenesis is typically studied at antibiotic concentrations close to, but below, the minimum inhibitory concentration (MIC). The evolutionary dynamics at play within this near-MIC regime are more complex than those that occur at lethal concentrations of antibiotic (**Figure 1**). At concentrations near the MIC, selection for resistant variants will be weaker than for concentrations above MIC, although it is important to note that many antibiotics remain selective at concentrations far below the MIC (Andersson and Hughes, 2014). Near to the MIC, population genetics will play a major role in determining evolutionary outcomes (Hughes and Andersson, 2017). Competition for resources

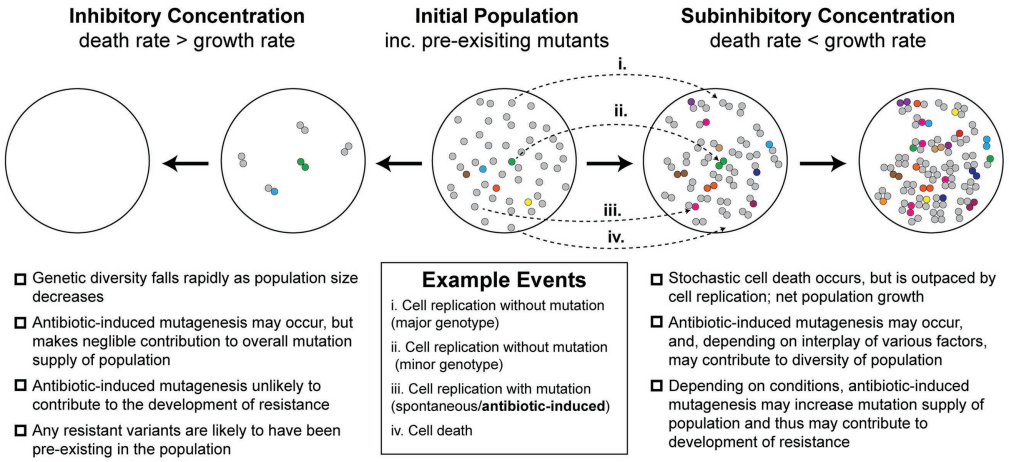

FIGURE 1 | Antibiotic-induced mutagenesis is one of many factors that influence genetic diversity in bacterial populations. Many antibiotics induce elevated rates of mutagenesis in bacterial cells. However, the relative importance of antibiotic-induced mutagenesis in shaping the evolution of antibiotic resistance remains unclear. This figure illustrates some of the competing factors that increase and decrease genetic diversity (i.e., number of unique mutants) in antibiotic-exposed bacteria. It is not intended to serve as a functional model of resistance development. An initial population of antibiotic-naïve cells appears in the center. The majority of cells in the population are of the major genotype ("wild type"; gray circles). Due to there being a basal rate of spontaneous mutagenesis, some cells have variant genotypes (colored circles). The left panels depict a scenario in which a lethal dose of bactericidal antibiotics is administered. Under these conditions, the rate of cell death outpaces the rate of cell division. In the first stage of this inhibitory concentration scenario, most of the population is killed (circles disappear). A few cells initially survive long enough to divide, however, in time these are also killed. Thus, no cells remain alive by the second stage of the scenario. Because there is little cell growth, there is little scope for antibiotic-induced mutagenesis. The genetic diversity of the population crashes as rapid cell death dominates over other factors. Any antibiotic-resistant mutants that are selected under these conditions are likely to be pre-existing in the population prior to antibiotic exposure. In the second scenario, depicted in the two right panels, a sub-inhibitory concentration of a bactericidal antibiotic is applied. As demonstrated recently (Coates et al., 2018), many cells die under these conditions, however, the rate of cell division outpaces the rate of cell death, leading to net population growth. In the first stage of this sub-inhibitory concentration scenario, a portion of cells die (~30%), while the remaining cells divide (~70%). Antibiotic-induced mutagenesis increases the probability that variants are produced during these cell divisions (gray circles more often yield colored circles). Specific examples of each event are depicted with arrows (i–iv; see example events legend). These processes of stochastic cell death, cell division, and mutagenesis are repeated in the second stage. Over time, the population becomes larger and contains a greater number of variants than the initial population. It is important to note, however, that the number of cell divisions required to reach a particular population size will be higher than what would be required in the absence of drug. Many experimental measures of induced mutagenesis rates neglect this behavior and will therefore be overestimated if cell death is occurring frequently (Frenoy and Bonhoeffer, 2018). Nevertheless, antibiotic-induced mutagenesis can significantly contribute to the development of antibiotic resistance *in vivo* (Cirz et al., 2005). Overall, the genetic diversity of the population in the sub-inhibitory concentration scenario will depend on the complex interplay between cell death, cell division, spontaneous mutagenesis, and induced mutagenesis. Selection for antibiotic-resistant phenotypes will occur at all stages and is likely to involve additional complexity arising from population-level phenomena that are not depicted in the figure.

between variants (clonal interference) will play a large role in determining the population structure (Hughes and Andersson, 2017). Population size will also shape evolutionary outcomes – large populations tend to disfavor the selection of rare variants unless they are particularly advantageous (Hughes and Andersson, 2017). The relative rates of cell growth and cell death will also be important (Coates et al., 2018). It is widely known that exposing cells to near-MIC concentrations of bactericidal antibiotics causes the population to grow at a diminished rate. However, it was only demonstrated recently that this occurs because a portion of the population undergoes stochastic cell death (Coates et al., 2018). Thus, the population growth rate slows because the cell death rate approaches the cell growth rate, rather than all the cells simply growing at a slower rate. This phenomenon is depicted in **Figure 1** and is expanded upon in a later section. At the same time that antibiotic-induced mutagenesis is acting to increase genetic diversity (i.e., number of unique mutants) within the population, cell death acts to reduce the size of the population. Evolutionary outcomes, including the likelihood of the population becoming antibiotic resistant, will depend strongly on the balance of these two parameters.

We propose that to better understand the role of antibiotic-induced mutagenesis on the evolution of antibiotic-resistance mutations, we need to be able to measure its effects while simultaneously monitoring cell and population level behaviors. This is not achievable with conventional microbiology techniques (e.g., fluctuation analyses), which operate as end-point assays and have indirect read-outs.

In recent years, researchers have begun to explore microscopy as a means to obtain direct, real-time observations of individual cells, and even individual molecules within them. The purpose of the current review is to highlight studies in which microscopy has improved our understanding of either the molecular mechanisms underlying antibiotic-induced mutagenesis or cell- and population-level events that occur when bacteria are challenged with sub-inhibitory concentrations of antibiotics. Much of the microscopy work presented here has been conducted in *E. coli*, due to its prevalence as a laboratory model organism, and with fluoroquinolones because these drugs are strongly

mutagenic and are commonly utilized as model compounds in laboratory experiments. For a more comprehensive review of antibiotic-induced mutagenesis, which includes work in other systems, we suggest a recent review by Blazquez et al. (2018). There remains tremendous scope to extend the studies highlighted within this review to other organisms and other antibiotics. While microscopy cannot resolve all of the gaps in knowledge that remain open in the field of antibiotic-induced mutagenesis, the studies highlighted in the current review demonstrate the potential for enormous gains to be made. We anticipate that the greatest impact of microscopy will be in improving our understanding of how mutation rates are modulated in bacterial cells and in mapping mutations to fitness effects.

THE CELLULAR CONTEXT OF ANTIBIOTIC-INDUCED MUTAGENESIS

Before discussing the molecular mechanisms of antibiotic-induced mutagenesis, it is useful to consider the cellular context in which it takes place. Elevated mutagenesis is induced by several classical DNA-damaging agents, including UV light, mitomycin c, and methyl methanesulfonate (Maslowska et al., 2019). It is likely that all the antibiotics that induce mutagenesis do so by causing DNA damage. This can occur as a direct consequence of their mode of action (as is the case for fluoroquinolones, which inhibit type II-topoisomerases). Alternatively, DNA damage can occur indirectly, through errant metabolic processes that generate reactive-oxygen species (ROS; Kohanski et al., 2010). In **Figure 2**, we summarize some of the events known to take place in *E. coli* cells treated with ciprofloxacin, a fluoroquinolone antibiotic that is a key model compound in the study of antibiotic-induced mutagenesis (Cirz et al., 2005; Zhang et al., 2011; Bos et al., 2015; Pribis et al., 2019). It is likely that much of what we summarize for *E. coli* cells treated with ciprofloxacin extends to other organisms and to other antibiotics, although in most cases this remains to be tested.

Broadly speaking, *E. coli* cells respond to ciprofloxacin the same way they do to sub-inhibitory doses of other DNA damaging agents – they induce the SOS response (Pribis et al., 2019). SOS induction has been measured directly for several fluoroquinolones (Ysern et al., 1990; Thi et al., 2011; Pribis et al., 2019; Rodriguez-Rosado et al., 2019), as well as for the antifolates trimethoprim and sulfamethoxazole (Thi et al., 2011; Blazquez et al., 2012), and representative members of the β-lactams (Thi et al., 2011; Blazquez et al., 2012). Microscopy has revealed much about how *E. coli* cells respond to ciprofloxacin treatment. Once exposed, the cells continue to grow, but divide much less often, causing them to form long filaments (**Figures 2A,B**; Diver and Wise, 1986; Thi et al., 2011; Bos et al., 2015; Henrikus et al., 2018; Pribis et al., 2019). Bulk DNA replication continues during sub-inhibitory ciprofloxacin treatment (likely at a diminished rate; Goss et al., 1965; Snyder and Drlica, 1979) and as a result the filamentous cells contain multiple nucleoid masses (**Figure 2C**; Bos et al., 2015). This suggests that filamentous cells contain multiple independent (or semi-independent) copies of the bacterial chromosome, although it is important to note that the masses are less organized than the nucleoids of untreated cells, and the study did not examine whether masses represent intact or partially degraded nucleoids.

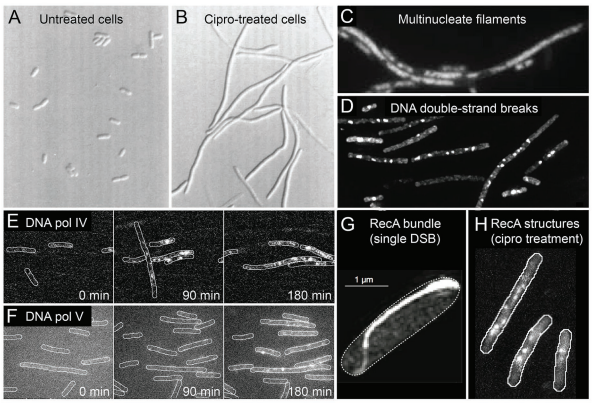

FIGURE 2 | Conditions inside bacterial cells undergoing antibiotic-induced mutagenesis. With the exception of panels **(A)** and **(G)**, all panels depict *Escherichia coli* cells exposed to ciprofloxacin, a bactericidal antibiotic of the fluoroquinolones class. **(A,B)** *E. coli* growing as rod-shaped cells in the absence of antibiotics **(A)** and as filamentous cells in the presence of ciprofloxacin **(B)**. Figure adapted from Diver and Wise (1986). **(C)** Filamentous *E. coli* cells contain multiple copies of the chromosome, as shown by DAPI staining. The number of chromosomes scales with filament length. Figure adapted from Bos et al. (2015). **(D)** Large numbers of DNA double-strand breaks in ciprofloxacin-treated cells, as detected by a fluorescent protein fusion of the Gam protein. Figure adapted from Pribis et al. (2019). **(E,F)** Increased activity of fluorescently labeled error-prone DNA polymerases IV **(E)** and V **(F)** in ciprofloxacin-treated cells, as visualized by single-molecule fluorescence microscopy. Figures adapted from Henrikus et al. (2018, 2020). **(G)** Large bundle of fluorescently labeled RecA formed in an *E. coli* cell that contains a single DNA double-strand break induced *via* expression of the SceI restriction enzyme. Figure adapted from Lesterlin et al. (2014). **(H)** Colocalisation of error-prone DNA polymerase IV with RecA in ciprofloxacin-treated cells. RecA-ssDNA nucleoprotein filaments (RecA*) are specifically visualized using a portion of the RecA*-binding cI repressor protein that has been fused to a fluorescent protein. Figure adapted from Henrikus et al. (2020).

Cells treated with ciprofloxacin contain elevated numbers of DNA double-strand breaks, which can be detected using fluorescent fusions of the Gam protein from bacteriophage λ (**Figure 2D**; Shee et al., 2013; Pribis et al., 2019). Detection of double-strand breaks through a fluorescent protein fusion of the RecN protein has also been reported (Hong et al., 2017). It is likely that cells treated with other bactericidal compounds also contain large numbers of breaks as a consequence of having elevated ROS levels (Liu et al., 2010; Foti et al., 2012; Giroux et al., 2017). We have detected significant numbers of breaks appearing in cells treated with trimethoprim, for example (Henrikus et al., 2020). Double-strand breaks are important lesions for antibiotic-induced mutagenesis for multiple reasons. First, double-strand breaks are considered to be lethal to cells if left unrepaired (Cox, 2013). Second, the processing of double-strand breaks by the RecBCD helicase-nuclease complex is one of the major triggers for the induction of the SOS response in *E. coli* (Simmons et al., 2008). This probably extends to other organisms such as *Bacillus subtilis*, that uses the functional analog of RecBCD, AddAB (Simmons et al., 2008). Third, much of the increased mutagenesis associated with ciprofloxacin treatment in *E. coli* can be attributed

to a form of error-prone double-strand break repair (Pribis et al., 2019). This activity requires error-prone DNA polymerases IV and V, induction of the SOS response (to produce sufficient amounts of these polymerases), and induction of the RpoS response (described in detail later), which plays a crucial, but unidentified role. Using single-molecule fluorescence microscopy, the activation of pols IV and V in cells exposed to ciprofloxacin has now been observed directly (**Figures 2E,F** and section below; Henrikus et al., 2018). Fourth, homologous recombination is the primary mode of repair for double-strand breaks in *E. coli* (Dillingham and Kowalczykowski, 2008). The presence of multiple double-strand breaks in ciprofloxacin-treated cells (**Figure 2D**), together with the presence of multiple chromosome equivalents (**Figure 2C**), means that high levels of homologous recombination are likely to take place between chromosome equivalents. Inter-chromosomal homologous recombination is thought to be mediated through the formation of large structures by the RecA protein (**Figures 2G,H**; Levin-Zaidman et al., 2000; Renzette et al., 2005; Lesterlin et al., 2014; Ghodke et al., 2019). Inter-chromosomal recombination has the potential to remove mutations, to introduce mutations (through error-prone break repair), and to combine mutations that originate within different chromosome variants into a single chromosome (Guttman and Dykhuizen, 1994; Lopez et al., 2007; Lopez and Blazquez, 2009; Pribis et al., 2019). In *E. coli*, inter-chromosomal recombination is strongly stimulated by exposure to ciprofloxacin (Lopez et al., 2007; Lopez and Blazquez, 2009), but not by antibiotics outside of the fluoroquinolone class (Lopez and Blazquez, 2009).

While the phenomena described above provide valuable insight into events in cells that accompany antibiotic-induced mutagenesis, they are by no means the only factors involved. Environmental factors, including media composition, can dramatically impact mutagenesis (Maharjan and Ferenci, 2017). The intracellular production of ROS is demonstrated to be important for mutagenesis induced by ciprofloxacin (Pribis et al., 2019; Rodriguez-Rosado et al., 2019) and is highly likely to be involved in mutagenesis induced by other antibiotics (Kohanski et al., 2010; Thi et al., 2011). Interestingly, while ciprofloxacin-induced mutagenesis is largely ROS-dependent (Pribis et al., 2019; Rodriguez-Rosado et al., 2019), the mutation signatures produced do not match the expected signature for incorporation of 8-oxoguanine, an aberrant nucleotide that is highly mutagenic toward DNA replication *in vitro* and is known to accumulate in high ROS cells (Song et al., 2016). This suggests that the primary contribution of ROS production toward ciprofloxacin-induced mutagenesis is the triggering of mutagenic DNA repair pathways, as opposed to mutagenesis that results directly from oxidation of the nucleotide pool.

MOLECULAR MECHANISMS OF ANTIBIOTIC-INDUCED MUTAGENESIS: ACTIVATION OF SOS AND ERROR-PRONE DNA POLYMERASES

The most widely studied form of antibiotic-induced mutagenesis is associated with the SOS response and involves increased production of error-prone DNA polymerases. *E. coli* produce three SOS-induced DNA polymerases (pols), pol II, pol IV, and pol V (Goodman and Woodgate, 2013). Of these, pols IV and V appear to be most responsible for SOS-dependent antibiotic-induced mutagenesis, although pol II can contribute to mutagenesis in some circumstances (Cirz et al., 2005; Pribis et al., 2019).

Pol V is the most error-prone DNA pol produced in *E. coli* cells (Jaszczur et al., 2016). Its activity is regulated at multiple levels, ensuring that its activity in the absence of exogenous DNA damage is minimal (Goodman et al., 2016). At least three steps in the pol V regulation pathway are dependent on RecA* nucleoprotein filaments (multimeric filaments of RecA on single-stranded DNA), which are produced during the repair of single-stranded DNA gaps and double-strand breaks *via* homologous recombination (Goodman et al., 2016). First, pol V is assembled from the products of an SOS-regulated operon, *umuDC* (Tang et al., 1999; Fernandez de Henestrosa et al., 2000). Induction of the SOS response occurs when RecA* nucleoprotein filaments induce autocatalytic cleavage of the SOS repressor protein LexA (Maslowska et al., 2019). Thus, the $UmuD_2$ (UmuD homodimer) and UmuC proteins are only expressed once RecA* accumulates in cells. At this stage, however, $UmuD_2$ and UmuC do not form an active polymerase. Second, $UmuD_2$ homodimers undergo autocatalytic cleavage in the presence of RecA*, in a mechanism analogous to that of LexA (Goodman et al., 2016). This cleavage produces the shorter protein $UmuD'_2$. Whereas $UmuD_2$ and $UmuD'_2$ are relatively stable, the intermediate heterodimer form UmuDD' is rapidly degraded by the ClpXP protease, which imparts a form of threshold on the RecA*-mediated conversion of $UmuD_2$ to $UmuD'_2$ (Gonzalez et al., 1998). Third, RecA* is again required to form the active version of pol V, known as pol V Mut. $UmuD'_2$ and UmuC physically interact to form pol V; however, this complex is a very poor polymerase (note that in this context pol V specifically refers to the complex $UmuD'_2C$; Goodman et al., 2016). In a remarkable feat of biochemistry, pol V next locates a RecA* filament and extracts a single RecA protomer, with ATP intact, from the 3' proximal end (Goodman et al., 2016). This process forms the mutagenically active complex pol V Mut ($UmuD'_2C$-RecA-ATP; Jiang et al., 2009). Once formed, pol V Mut remains active for only a few seconds before cleaving the ATP (*via* intrinsic ATPase activity; an unprecedented function among polymerases; Erdem et al., 2014; Jaszczur et al., 2019), at which point the complex becomes inactive (Goodman et al., 2016).

Microscopy is already beginning to play a major role in improving our understanding of these polymerases in bacteria (Joseph and Badrinarayanan, 2020). Using single-molecule fluorescence microscopy, we visualized the RecA*-dependent activation of pol V in cells treated with DNA damaging agents, including ciprofloxacin (Robinson et al., 2015; Henrikus et al., 2020). In doing so, we discovered yet another element that controls pol V activity – spatial regulation (**Figure 3A**; Robinson et al., 2015). While spatial regulation has been well-established in eukaryotic systems, this was the first example of a DNA-acting enzyme being spatially regulated in bacteria. In the case of

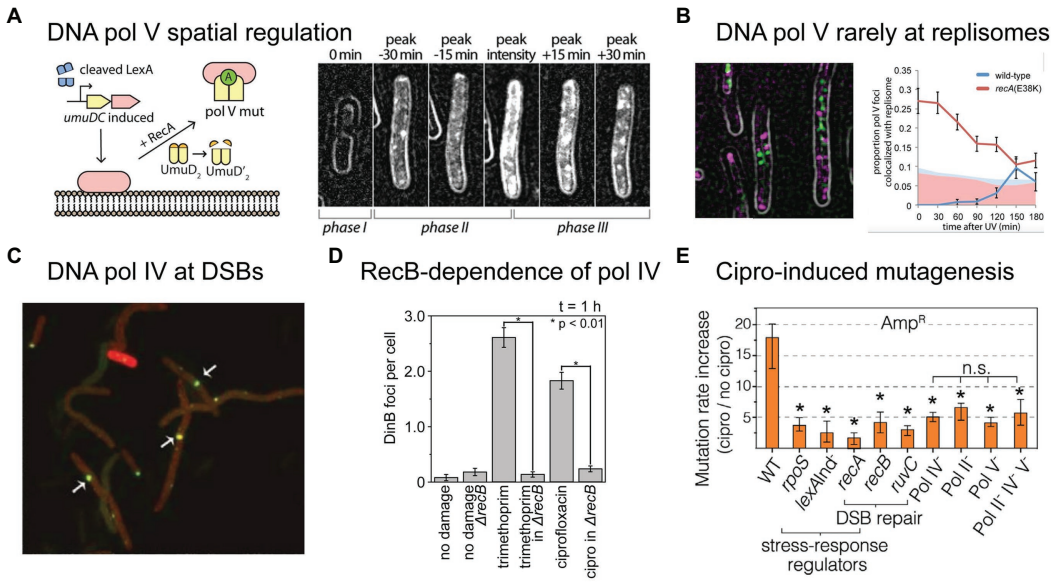

FIGURE 3 | Recent insights into the activities of error-prone DNA polymerases in bacterial cells. **(A,B)** Single-molecule fluorescence microscopy analysis of DNA polymerase V in *E. coli* cells carrying UV-induced DNA damage. Figures adapted from Robinson et al. (2015). **(A)** DNA polymerase V is a rare example of a spatially regulated polymerase enzyme in bacteria, with intermediate forms being localized to the cell membrane and only the fully activated form being allowed to redistribute into the cytosol. The active form of DNA polymerase V, pol V Mut, is comprised of two molecules of UmuD', one molecule of UmuC, one molecule of RecA, and one molecule of ATP (i.e., UmuD'$_2$-UmuC-RecA-ATP; Jiang et al., 2009). The formation of pol V Mut is regulated at several levels, in order to minimize pol V-dependent mutagenesis in the absence of DNA damage (Goodman et al., 2016). All stages of pol V regulation involve activities of RecA* nucleoprotein filaments. A single-molecule fluorescence microscopy analysis of cells expressing a fluorescent protein fusion of UmuC (UmuC-mKate2), revealed three stages in the activation of pol V in cells treated with a burst of UV light (Robinson et al., 2015). In phase I, cells began to grow into filaments, indicating induction of the SOS response, but produced little UmuC-mKate2. In phase II, cells began to express UmuC-mKate2. In this phase, UmuC-mKate2 was localized to the cell membrane. Due to the small number of UmuC-mKate2 features present in each cell, this can be difficult to see in individual representative images, but becomes abundantly clear in statistical analyses of cells across multiple images. Membrane localization is most evident in the image labeled "peak intensity", where clear membrane-associated signals are visible along the edge of the cell. Finally, in phase III, UmuC-mKate2 was released into the cytosol as pol V Mut, where it formed punctate foci on the nucleoid, indicative of DNA synthesis occurring. This can be seen most clearly in the image labeled "peak + 30 min". Release of UmuC from the membrane requires the cleavage of the UmuD protein to its shorter form UmuD'. **(B)** Pol V Mut rarely colocalises with replisomes. Contrary to existing models of pol V-dependent DNA synthesis, in which pol V carries out translesion synthesis at stalled replication forks, single-molecule fluorescence imaging revealed that pol V Mut rarely colocalises with replication forks. This indicates that pol V likely works on other DNA substrates in cells, which may include single-stranded gaps and/or recombination intermediates. **(C–E)** DNA polymerase IV (pol IV) works on double-strand break repair intermediates in cells treated with ciprofloxacin. **(C)** Single-molecule fluorescence microscopy demonstrated that fluorescently labeled pol IV forms foci that colocalise with sites of double-strand breaks induced by the SceI restriction enzyme. Figure adapted from Mallik et al. (2015). **(D)** A second single-molecule imaging study demonstrated that the formation of pol IV foci in cells treated with either ciprofloxacin or trimethoprim requires processing of double-strand breaks by the RecBCD complex. Figure adapted from Henrikus et al. (2020). This, together with other evidence, indicates that these foci likely represent pol IV working on recombination intermediates during double-strand break repair. **(E)** Ciprofloxacin-induced mutagenesis is a form of error-prone double-strand break repair, requiring induction of the SOS and RpoS responses (regulated by *lexA* and *rpoS*), double-strand break repair (dependent on *recA*, *recB*, *recC*, and *ruvC*), and error-prone DNA polymerases (Pols II, IV, and V). Figure adapted from Pribis et al. (2019). Crucially, the RpoS response is only induced in a sub-population of cells, indicating that ciprofloxacin-induced mutation rates are elevated within only a fraction of the cell population (~10%).

pol V, spatial regulation is imposed *via* an interaction between UmuC and the inner cell membrane. Following SOS-induced expression of UmuD$_2$ and UmuC, but prior to UmuD′$_2$ being formed, UmuC associates with the membrane. Once UmuD′$_2$ is formed, UmuC is released from the membrane as part of either pol V (UmuD′$_2$C) or pol V Mut (UmuD′$_2$C-RecA-ATP). It remains unknown whether pol V Mut formation occurs on the membrane or in the cytosol. In the microscopy images, pol V spatial regulation presents as a redistribution of fluorescently labeled UmuC from the membrane to the cytosol. Due to the fact that UmuC is expressed at very low levels in *E. coli* (0–15 mol/cell; Robinson et al., 2015), UmuC produced patchy signals and this spatial redistribution was difficult to observe by eye, but was well-supported by statistical analyses. In subsequent work, we developed a novel image-analysis technique that enabled us to extract the relative proportion of membrane-bound and cytosolic UmuC as a function of time (Goudsmits et al., 2016). As UmuC levels peak, 90% of the protein is associated with the membrane. Within 30 min, this proportion decreases to 50%, with the remaining 50% now appearing in the cytosol. As pol V Mut is formed and begins to work on the DNA within the cytosol, punctate foci of UmuC are formed. This phenomenon is known as detection by localization and occurs because the rate of diffusional motion slows dramatically once the protein binds to the DNA. Interestingly, we observed that pol V Mut foci formed at sites on the nucleoid that were spatially distinct from replisomes (**Figure 3B**). This strongly suggests that pol V Mut acts on

non-replisomal substrates, such as single-stranded DNA gaps or homologous recombination intermediates (Robinson et al., 2015). In a follow-up study, we observed that the number of pol V Mut foci formed in ciprofloxacin-treated cells did not appear to correlate with the number of double-strand breaks present, indicating that the substrates for pol V Mut are unlikely to be homologous recombination intermediates formed during double-strand break repair (Henrikus et al., 2020).

In contrast, recent studies have revealed a clear role in double-strand break repair for the other error-prone polymerase present in *E. coli*, pol IV. A role for pol IV in (error-prone) break repair was originally discovered through genetics experiments (Ponder et al., 2005). The single-molecule imaging measurements described below demonstrated that break-repair intermediates are the major substrates for pol IV in cells treated with (fluoro)quinolone antibiotics.

While many had tried, the Foster group was the first to produce a functional fluorescent protein fusion of pol IV (Mallik et al., 2015). This was a considerable achievement as assaying for pol IV-dependent activities in cells is non-trivial. Furthermore, the activity of fusions turns out to be highly sensitive to the length of the linker used to connect the pol IV protein (DinB) to the fluorescent protein. Using the active (20 amino-acid linker) fusion they showed, among other things, that pol IV localizes as punctate foci in cells treated with the quinolone antibiotic naladixic acid (Mallik et al., 2015). These foci tightly colocalised with RecA* foci, which are expected to form at sites of double-strand breaks. Using a restriction enzyme to induce a single break within the chromosome, they observed that pol IV localized to the break site (**Figure 3C**). Building on this work, we showed that the binding of pol IV to the nucleoid in cells treated with ciprofloxacin or trimethoprim was strongly dependent on a gene (*recB*) involved in double-strand break repair (Henrikus et al., 2020). Disruption of the RecBCD complex, which carries out the early stages of double-strand break repair, almost completely abolished the formation of pol IV foci in cells treated with either ciprofloxacin or trimethoprim (**Figure 3D**). Together with existing evidence from genetics and biochemistry studies (Ponder et al., 2005; Williams et al., 2010; Shee et al., 2011a,b, 2012; Pomerantz et al., 2013a,b; Tashjian et al., 2019), these microscopy observations indicated that a major role of pol IV in cells is to act in double-strand break repair. The likely function for pol IV in this context is the extension of D-loop structures.

Using a combination of microscopy and classical genetics approaches, the Rosenberg group recently demonstrated that ciprofloxacin-induced mutagenesis is ROS-dependent, and is driven by error-prone double-strand break repair (Pribis et al., 2019). Ciprofloxacin-induced mutagenesis was found to be dependent on genes encoding pols II, IV, and V (*polB*, *dinB*, and *umuDC*), double-strand break repair factors (*recA*, *recB*, and *ruvC*), the SOS response (mutagenesis was blocked in a *lexA* [Ind$^-$] background), and the RpoS response (**Figure 3E**; the RpoS response is introduced in greater detail in the next section). The authors discovered that ROS levels were elevated in a sub-population (~10%) of ciprofloxacin-treated cells. Elevated mutagenesis was strongly enriched within this sub-population. In this context, the main function of ROS is to induce the RpoS response; mild overexpression of RpoS alleviated the requirement for ROS in the mutagenesis assays. The observation of an RpoS-high sub-population is an example of phenotypic heterogeneity. Microscopy is revealing phenotypic heterogeneity to be a common theme bacterial DNA repair and mutagenesis (Vincent and Uphoff, 2020). Improving our understanding of population heterogeneities is vital if we are to better understand antibiotic-induced mutagenesis.

Whereas, the Rosenberg study points to pol IV-dependent error-prone break repair occurring in a subset of high-ROS, RpoS-positive cells (Pribis et al., 2019), our imaging study showed that ciprofloxacin-induced pol IV foci formed in all cells (Henrikus et al., 2020). The model that is most consistent with the data is that pol IV is involved in double-strand break repair in all cells and that this somehow becomes error-prone in the RpoS-positive sub-population. In other words, the RpoS response modulates the fidelity of pol IV, rather than its ability to engage double-strand break repair intermediates. The role of RpoS in mutagenesis remains unknown, however, it is tempting to speculate that RpoS-dependent modulation of mismatch repair could play a role, as has been reported in other systems (Gutierrez et al., 2013).

MOLECULAR MECHANISMS OF ANTIBIOTIC-INDUCED MUTAGENESIS: RpoS RESPONSE AND REGULATION OF MISMATCH REPAIR

Using classical genetics approaches, the Matic group discovered that cells treated with the β-lactam antibiotic ampicillin induced pol IV-dependent mutagenesis (Gutierrez et al., 2013). As with the ciprofloxacin-induced mutagenesis (Pribis et al., 2019) described in the previous section, the RpoS response was found to play a key role in ampicillin-induced mutagenesis (Gutierrez et al., 2013). The RpoS response (also known as the σ^S response or general stress response) is induced under a variety of circumstances, including stationary growth, starvation, and oxidative damage (Battesti et al., 2011; Gottesman, 2019). The protein RpoS (or σ^S) is an alternative σ-factor that governs the expression of hundreds of different genes (Battesti et al., 2011). When expressed, RpoS competes with the housekeeping σ-factor, σ^{70} (or RpoD), for binding to RNA polymerase, and in doing so dramatically changes the gene expression profile of the cell (Battesti et al., 2011).

The Matic group showed that representative quinolone, β-lactam, and aminoglycoside antibiotics induced the RpoS response (Gutierrez et al., 2013). Choosing to investigate ampicillin in greater depth, they demonstrated that ampicillin-induced mutagenesis required both pol IV and induction of the RpoS response. Unlike ciprofloxacin-induced mutagenesis, induction of the SOS response was not required for ampicillin-induced mutagenesis. They also observed that mild overexpression of the mismatch repair protein MutS (which functions to recognize nucleotide mismatches) completely suppressed

mutagenesis. This led to the discovery of a small regulatory RNA, SdsR, which is expressed during the RpoS response and reduces the expression of MutS. They proposed a model in which depletion of mismatch repair leads to less efficient repair of mismatches produced by pol IV, which in turn leads to increased mutagenesis.

Representative quinolones and aminoglycosides also induce the RpoS response (Gutierrez et al., 2013) and exhibit pol IV-dependent mutagenesis (Thi et al., 2011). It is reasonable to speculate that the depletion-of-mismatch-repair mechanism at play during ampicillin-induced mutagenesis may extend to other bactericidal antibiotics. A single-molecule/single-cell imaging approach that would seemingly provide the ideal platform on which to test this idea has already been developed.

Elez developed an approach that allowed mismatches to be visualized by microscopy (Elez et al., 2012). By fluorescently labeling the mismatch repair protein MutL (the molecular coordinator of mismatch repair), it became possible to visualize the process of mismatch detection by the MutS and MutL proteins. Each time a mismatch was detected, a punctate MutL focus became visible in microscope images. In a series of later studies, Elez and colleagues repeated the measurements in cells that lacked the *mutH* gene, which is required for repair of the mismatch (Elez et al., 2010, 2018; Robert et al., 2018). This ensured that the mismatch would be fixed to a mutation in the next round of DNA replication, providing a means to infer mutation rates in real-time.

While it was not the primary goal his study (Uphoff, 2018), Stephan Uphoff used the labeled-MutL system to study mismatch-detection rates in cells treated with the fluoroquinolone antibiotic norfloxacin (**Figure 4**). Growing the cells in a single-cell chemostat device called a "mother machine" (**Figure 4A**; Wang et al., 2010), he observed a significant increase in the number of mismatches detected in cells by fluorescent MutL (Uphoff, 2018). Delivering a pulse of norfloxacin at a concentration equal to the MIC, a transient 5-fold increase in the number of detected mismatches was observed (**Figure 4B**). Delivering a lower concentration (0.25x MIC) for a longer time led to a more sustained elevation (3-fold) of the mismatch-detection rate. In both experiments, induction of the SOS was also measured, revealing that changes in the mismatch-detection rate correlated with induction of the SOS response. The MutL-based mismatch-detection system could be an extremely useful tool in the study of antibiotic-induced mutagenesis. In particular, it would allow the role of RpoS-based regulation of mismatch repair to be assessed much more broadly and at an unprecedented level of detail. Each MutL focus represents a mismatch that would normally be repaired before becoming fixed as a mutation. One would hypothesize that disruption of the RpoS response would prevent the down-regulation of MutS, leading to more efficient detection of mismatches and a corresponding increase in the number of MutL foci detected in cells treated with antibiotics.

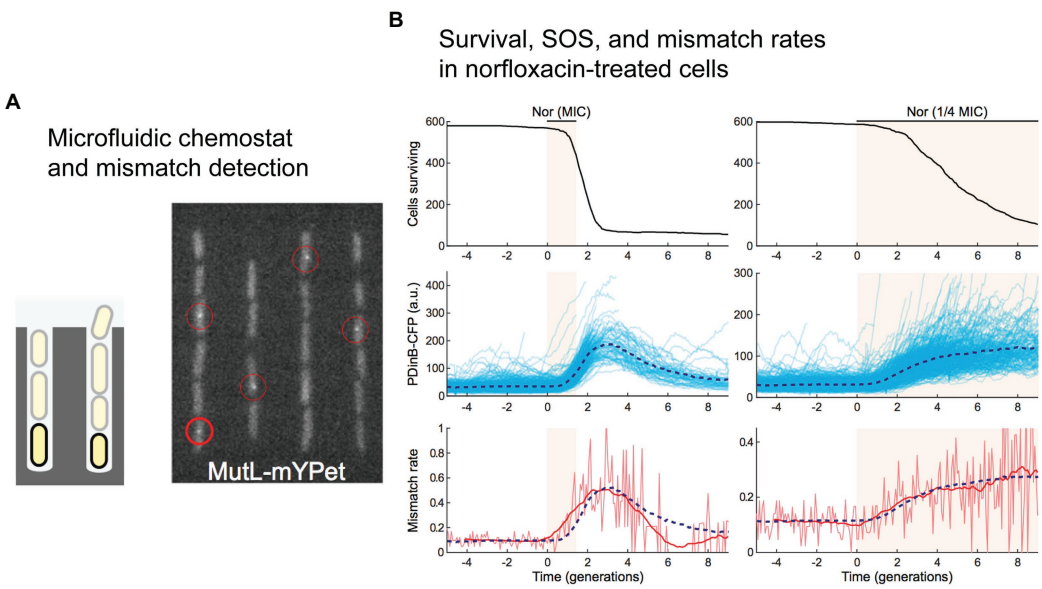

FIGURE 4 | Direct detection of mismatches in *E. coli* cells treated with norfloxacin. **(A,B)** A single-molecule fluorescence microscopy study of cells growing in microfluidic chemostats revealed an increased rate of mismatches in cells treated with the fluoroquinolone antibiotic norfloxacin. Figures adapted from Uphoff (2018). **(A)** A microfluidic "mother machine" was used to monitor individual cells through multiple generations. A fluorescent protein fusion of the mismatch repair protein MutL (MutL-mYPet) allowed mismatches to be visualized as punctate foci within cells. **(B)** Treatment of cells with norfloxacin induces a substantial increase in mismatch rate. Left unrepaired, these mismatches would become mutations during the subsequent round of DNA replication. The dynamics of mismatch formation match well with induction of the SOS response, which is monitored in this study by expression of cyan fluorescent protein (CFP) from the SOS-inducible *dinB* promoter (pDinB-CFP). Importantly, both SOS induction and the elevated mismatch rate lag behind cell death – increased mutagenesis is likely to occur in a small number of cells that survive beyond the initial stages of treatment.

MEASUREMENTS LINKING MUTATIONS TO CELL AND POPULATION OUTCOMES

The mismatch-detection approach developed by Elez would also be an ideal platform to begin connecting the molecular world of mutagenesis with changes at the cell and population levels. In fact, her group has already taken steps in this direction, measuring a key population-level descriptor, the distribution of fitness effects, and for cells growing in microfluidic mother machines (Robert et al., 2018). These devices allow for long-term measurements to be made under precisely controlled experimental conditions. The group monitored mismatch detection by MutL in a mismatch repair-defective *mutH* strain so that mismatches would be fixed as mutations in subsequent round of DNA replication (**Figure 5A**). In parallel, they tracked cell growth rates, enabling them to link the appearance of spontaneous mutations in cells with changes in growth rate. Because the cells were physically isolated from each other within the mother machine, there was no competition between variants in the population and thus, growth rate measurements directly reflected the fitness of the mutants in the conditions tested. Approximately, ~20,000 individual mutation events were detected over 200 generations. The measurements revealed that the majority of mutations observed in this experiment were quasi-neutral, having little, or no effect on fitness. Only 1% of mutations were lethal.

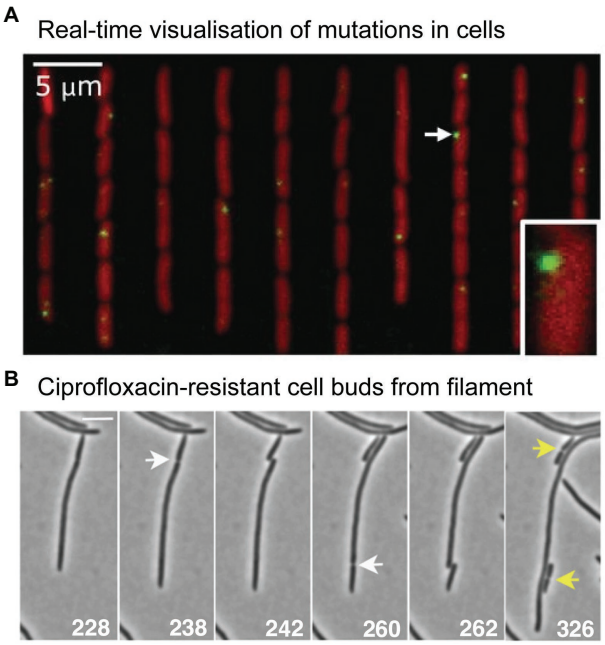

FIGURE 5 | Microscopy measurements linking mutations with phenotypic changes. **(A)** Direct measurement of a distribution of fitness effects from *E. coli* cells growing in a microfluidic chemostat. Figure adapted from Robert et al. (2018). Mismatches were detected using a fluorescent protein fusion of the mismatch repair protein MutL (YFP-MutL). Foci were produced in cells whenever YFP-MutL bound to a mismatch. The cells also carried a deletion of the *mutH* mismatch repair gene, ensuring that all mismatches became mutations in the subsequent round of DNA replication. The experimenters were able to link the appearance of mutations with changes in cell growth rates. They were able to produce a well-sampled distribution of fitness effects. Contrary to the dogma that most mutations are deleterious, the distribution showed that the vast mutations were quasi-neutral, having very little effect on growth. Only 1% of mutations were lethal. **(B)** Time-lapse microscopy captures the moment a ciprofloxacin-resistant cell buds from filamentous, multinucleate *E. coli* cell growing in a sub-inhibitory concentration of ciprofloxacin. Figure adapted from Bos et al. (2015).

Accurately measured distributions of fitness effects are key to evolutionary modeling and would be particularly advantageous in the modeling of antibiotic-resistance development. If the mother machine format could be adapted for use with antibiotics, this might even represent a route toward a quantitative assessment of the role of antibiotic-induced mutagenesis in the evolution of antibiotic resistance. Because the technique is based on microscopy, multiple facets of antibiotic-induced mutagenesis could be monitored in parallel. In addition to mismatch rates and cell growth rates, cell filamentation and cell death could also be monitored. With appropriate probes already in hand, it should be also possible to observe induction of the SOS and RpoS responses and corresponding activities of error-prone pols IV and V.

What might evolution to antibiotic resistance look like under the microscope? A 2015 study by Bos et al. (2015) provides some clues. As highlighted earlier (**Figure 2**), the authors observed that cells treated with ciprofloxacin at 0.125x MIC grow into filaments that contain multiple nucleoid masses (Bos et al., 2015). Occasionally, the authors observed regular-sized cells budding from the tip of these filaments (**Figure 5B**). Most of the time these buds were inviable, however, buds were occasionally produced that grew normally. These cells did not grow into filaments, indicating that they had become resistant to the antibiotic. Expansion of these resistant variants at 0.5x MIC or 1x MIC ciprofloxacin, followed by sequencing of the *gyrA* and *marR* loci, confirmed the presence of resistance mutations in many of the variants (in other cases, resistance was assumed to occur *via* mutations at other loci that were not sequenced). Expansion at 1x MIC strongly favored the *gyrA* (S83A) mutation, which appeared in 7/8 isolates. The Bos et al. (2015) study indicates that antibiotic-resistant variants can appear frequently enough to be detected *via* microscopy.

MEASUREMENTS LINKING CELL GROWTH DYNAMICS TO POPULATION OUTCOMES

Antibiotics can induce mutagenesis, tolerance, and cell death. Evolution to antibiotic resistance is often limited by the mutation supply rate, which is a product of the cellular mutation rate and the size of the population (Hughes and Andersson, 2017). Elevated mutagenesis acts to increase the mutation supply, while cell death reduces it by decreasing the size of the population. Seeking to minimize the effects of cell death, antibiotic-induced mutagenesis is typically studied using sub-MIC concentrations of antibiotic. Importantly, it was demonstrated recently that significant cell death still occurs in this sub-MIC regime. Through a combination of microscopy, plate-reader

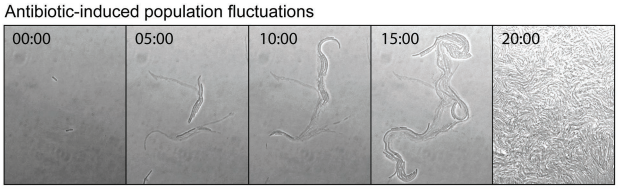

FIGURE 6 | Antibiotic-induced population fluctuations. It is well known that when bacterial growth is monitored as a function of antibiotic concentration, population growth rates tend to slow as the minimum inhibitory concentration (MIC) is approached. Recently, it was discovered that for bactericidal antibiotics this reduction in the net growth of the population is caused not by the slowing of cell division, but by some of the cells dying (Coates et al., 2018). As the MIC is approached, the rate of cell death approaches that of cell division. This is neatly visualized by a time-lapse movie, which shows cells undergoing stochastic cell death when growing at a concentration of cefsulodin equivalent to 0.8x MIC. Live and dead cells can be distinguished by contrast. The live cells produce high contrast in the bright-field images, whereas the dead cells, which have lysed and have left behind an empty cell-shaped sac, produce only low image contrast. At various points in the movie, a portion of the population undergoes stochastic cell death. These population fluctuations have far-reaching implications for the study of antibiotic-induced mutagenesis and for its potential to supply antibiotic resistance mutations. Figure adapted from Coates et al. (2018).

assays, and modeling, the Kim group investigated the extinction of small populations of cells (<100) upon treatment with antibiotics (Coates et al., 2018). Their analysis revealed that bactericidal antibiotics induced stochastic fluctuations in population size. This was visualized by time-lapse microscopy images of cells growing in the presence of the cephalosporin antibiotic cefsulodin, at a concentration equivalent to 0.8x MIC (**Figure 6**). During the course of the time-lapse measurement, the total number of cells that are present increases, however, this occurs more slowly than in the absence of drug because many of the cells die. Stochastic cell death leads to marked fluctuations in the population size as a function of time. This simple observation has broad ramifications for the study of antibiotic-induced mutagenesis.

The fact that population growth in this near-MIC regime is the product of both cell growth and cell death means that many more generations are required for the population to reach a certain size than would be required in the absence of drug. When rates of antibiotic-induced mutagenesis are measured using fluctuation analyses, the results are reported in units of mutations per genome per generation. Typically, the number of generations is calculated based on the population size after treatment, under the assumption that cells are not dying when the antibiotic concentration is below the MIC. In light of the stochastic population dynamics reported by the Kim group, it appears that many of the mutation rates reported in the literature may be overestimated because more generations occurred than were accounted for in the measurements (Frenoy and Bonhoeffer, 2018).

The second important ramification of stochastic population dynamics for the study of antibiotic-induced mutagenesis is that when the population size is small, as would be the case in a mother machine for example; many populations will become extinct when exposed to sub-MIC concentrations of bactericidal antibiotics. Evidence of this is visible in Uphoff's work with sub-MIC norfloxacin (Uphoff, 2018), where the number of viable cells decreases continuously throughout the experiment (**Figure 4B**). Presumably, this has occurred because of the small and finite size of the channels in the mother machine. Cells that are growing do not increase the size of the population being monitored because cells that no longer fit in the channel are washed away and thus are no longer monitored. Cells that die decrease the size of the observed population. Eventually, none of the channels would contain any viable cells. All of this occurred more quickly in cells treated with norfloxacin concentrations equivalent to the MIC because cells die more often. Because Uphoff monitored mismatch formation and cell death simultaneously we can carry out a putative assessment of the effects of norfloxacin exposure on mutation supply. In measurements with 1x MIC norfloxacin, mismatch-detection rates increased while cell viability fell (**Figure 4B**). At its peak, mismatch-detection rates were ~5x above basal levels (**Figure 4B**; lower left panel; three generation times). At this time point, however, survival had dropped to 10% of the original value (**Figure 4B**; upper left panel; three generation times). If we take the product of these two values as a crude approximation of the mutation supply rate within the mother machine device, mutation supply would have fallen by 50% in the norfloxacin-exposed population. In the measurements with 0.25x MIC norfloxacin, cell viability decreased more slowly (**Figure 4B**; upper right panel). By the five generations time-point, viability had reduced to 50% (**Figure 4B**; upper right panel), while the mismatch-detection rate had increased ~2.5x (**Figure 4B**; lower right panel). Thus, the mutation supply at this time would be predicted to be higher (125%) than that of the initial population. At a later time-point (eight generation times), the mutation supply would have been lower than the starting population; the mismatch-detection frequency had increased 3x, however, cell survival was only ~15%. The effects of cell death are amplified in the mother machine format, however, the fact that mismatch-detection frequencies and cell survival rates change as a function of time after drug exposure highlight the need for simultaneous, real-time measurements of mutation rates, and cell growth/death parameters. Such measurements are vital if we are to improve our understanding of antibiotic-induced mutagenesis. Bacterial population sizes are also affected by non-genetic phenomena, such as antibiotic tolerance and persistence. Accurate measurements of cell growth and survival would also provide new opportunities to assess the roles of antibiotic tolerance and persistence in the development of antibiotic resistance mutations.

CONCLUDING REMARKS

The studies highlighted throughout this review demonstrate the potential of microscopy in helping to achieve a more thorough understanding of antibiotic-induced mutagenesis. There is enormous potential for other types of correlative measurements as well. Microscopy shows a great degree of promise as an important platform for uncovering functional

linkages between the molecular-level actions of error-prone DNA repair processes, the cell-level behaviors of stress responses and growth dynamics, and population-level behaviors that determine mutation supply rates. The development of advanced techniques for microscopy and microfluidic technology is sure to continue, however, we feel it is important to point out that great strides can be taken with the technology that is already available. As has been noted previously (Potvin-Trottier et al., 2018), our understanding of other antibiotic-induced phenomena, including tolerance and persistence, would also strongly benefit from further application of state-of-the-art microscopy and microfluidic technologies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Transcriptomic Signature of Tigecycline in *Acinetobacter baumannii*

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Tigecycline, a protein translation inhibitor, is a treatment of last resort for infections caused by the opportunistic multidrug resistance human pathogen *Acinetobacter baumannii*. However, strains resistant to tigecycline were reported not long after its clinical introduction. Translation inhibitor antibiotics perturb ribosome function and induce the reduction of (p)ppGpp, an alarmone involved in the stringent response that negatively modulates ribosome production. Through RNA sequencing, this study revealed a significant reduction in the transcription of genes in citric acid cycle and cell respiration, suggesting tigecycline inhibits or slows down bacterial growth. Our results indicated that the drug-induced reduction of (p)ppGpp level promoted the production but diminished the degradation of ribosomes, which mitigates the translational inhibition effect by tigecycline. The reduction of (p)ppGpp also led to a decrease of transcription coupled nucleotide excision repair which likely increases the chances of development of tigecycline resistant mutants. Increased expression of genes linked to horizontal gene transfer were also observed. The most upregulated gene, *rtcB*, involving in RNA repair, is either a direct tigecycline stress response or is in response to the transcription de-repression of a toxin-antitoxin system. The most down-regulated genes encode two β-lactamases, which is a possible by-product of tigecycline-induced reduction in transcription of genes associated with peptidoglycan biogenesis. This transcriptomics study provides a global genetic view of why *A. baumannii* is able to rapidly develop tigecycline resistance.

Keywords: tigecycline, antibiotic resistance, *Acinetobacter baumannii*, transcriptomics, bacterial physiological response to antibiotics

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INTRODUCTION

Tigecycline is a broad-spectrum antibiotic derived from minocycline and was the first glycylcycline class antibiotic approved for clinical use (Petersen et al., 1999). Compared to tetracyclines, tigecycline has increased antibacterial potency due to its higher binding affinity with the 70S ribosomes, or more specifically with the helix 31 and 34 of the16S rRNA on the head of the 30S subunit (Jenner et al., 2013). This effect inhibits the delivery of the thermo-unstable ternary complex elongation factor (EF-Tu)·GTP·aminoacyl-tRNA to the ribosomal A (aminoacyl) site and eventually perturbs polypeptide translation (Jenner et al., 2013). Additionally, tigecycline is not

recognized by the major tetracycline resistance determinants, namely the major facilitator family (MFS) efflux pumps such as TetA/B which export tetracyclines out of the cell (Hirata et al., 2004), and ribosome protection proteins such as TetO and TetM which sequester tetracycline by binding to the tetracycline-stalled ribosome (Bergeron et al., 1996; Jenner et al., 2013).

Although tigecycline is not affected by common tetracycline resistance determinants, there have been increasing numbers of tigecycline resistant bacterial pathogens reported since its introduction in 2005. In Gram-negative organisms, the majority of such cases have been partially linked to the constitutive overexpression of resistance-nodulation-division (RND) efflux pumps, for instance MexXY-OprM in *Pseudomonas aeruginosa*, and AdeIJK and AdeABC in *Acinetobacter baumannii* (Dean et al., 2003; Visalli et al., 2003; Peleg et al., 2007; Damier-Piolle et al., 2008). Similarly, some Gram-positive organisms that display reduced susceptibility to tigecycline constitutively overexpress efflux pumps, such as the MATE family efflux pump MepA in *Staphylococcus aureus* (McAleese et al., 2005). Mutations of ribosomal protein genes and the 16S rRNA gene have also been shown to reduce tigecycline susceptibility of various organisms (Beabout et al., 2015; Lupien et al., 2015; Niebel et al., 2015), probably by affecting its target-site binding affinities. The proteobacterial TetX flavin-dependent monooxygenase, capable of inactivating tetracyclines, was also found to mediate tigecycline resistance when highly expressed (Moore et al., 2005). Plasmid- or mobile genetic element (MGE)-borne *tetX* genes have been identified in tigecycline resistant bacterial isolates from clinical and animal husbandry settings (He et al., 2019, 2020; Sun et al., 2019; Wang et al., 2019).

Acinetobacter baumannii is an opportunistic nosocomial human pathogen (Peleg et al., 2008). Tigecycline is one of the last resort therapies for the infections caused by carbapenem-resistant *Acinetobacter baumannii* strains, which have been listed as a top research priority for novel therapy development by the World Health Organization and the United States Centers for Disease Control and Prevention (CDC, 2019). Despite its broad spectrum antibacterial efficacy there have been reports of the development of resistant mutants during or after therapy and the United States Food and Drug Administration has warned of increased mortality risk for infections treated by tigecycline in comparison with other antibiotics (Navon-Venezia et al., 2007; Schafer et al., 2007; Anthony et al., 2008; Cai et al., 2011; Niu et al., 2019). The emergence of tigecycline resistant *A. baumannii* isolates can be correlated with a hypermutator phenotype (Hammerstrom et al., 2015). The plasmids or MGEs that carry *tetX* often also confer resistance to a wide range of other antibiotics (Morosini et al., 2006; Hornsey et al., 2011). Furthermore, the efflux pumps that can export tigecycline usually confer multidrug resistance (MDR) when overexpressed. Together these findings imply a future of tighter therapeutic options left for MDR *A. baumannii* infections or the emergence of pan-drug resistant infections. In this study, we sought to further characterize the genetic basis of tigecycline resistance in *A. baumannii*. To achieve this aim, RNA-Seq was used to analyse *A. baumannii* global transcriptomic response to tigecycline. We revealed that in addition to disrupting protein translation, tigecycline at a sub-inhibitory concentration also induced pleiotropic physiological effects, including differential expression of genes involved in RNA metabolism and DNA repair. These observations provide further insights into how *A. baumannii* may rapidly develop tigecycline resistance.

RESULTS AND DISCUSSION

A global clonal lineage I (GCI) *A. baumannii* 6772166 (Eijkelkamp et al., 2011; Hamidian and Hall, 2014) is an intermediate tigecycline resistant clinical isolate, with a minimum inhibitory concentration (MIC) between 2.5 and 5 μg/ml. When exposed to antimicrobials at a sub-inhibitory concentration, bacteria tend to evolve and develop antimicrobial resistance (Baym et al., 2016). In this study, we aimed to capture the physiological response to tigecycline in parental cells, rather than in the offspring population, which may be mixed by cells that have adapted to the drug. Because the *A. baumannii* doubling time at log phase is around 25 min and tigecycline slows down bacterial growth, this strain at mid-exponential growth phase was exposed to tigecycline at 2.5 μg/ml for 30 min, and the global transcriptomic response was analyzed via RNA-Seq.

Hua et al. has published similar work in a different *A. baumannii* clinical isolate, and their data revealed that tigecycline induces pleiotropic physiological impacts (Hua et al., 2014). Although not discussed in their paper, the data from Hua et al. (2014) also showed upregulation of genes encoding ribosomal proteins and drug efflux pumps, and downregulation of the genes involved in citric acid cycle and cell respiration chain. However, our current study provides additional physiological and biomolecular insights. We showed more than 1000 genes with a greater than two-fold change in gene expression following tigecycline exposure in *A. baumannii* 6772166 (**Supplementary Figure 2** and **Supplementary Data**), indicating that tigecycline induces broad physiological changes. This widespread alteration in gene transcription may be partially explained by the significant expression changes in 76 genes encoding putative or characterized transcriptional regulators, the up regulation of one highly expressed σ^{70} factor homolog gene, and the altered expression of various ribonuclease genes (**Figure 1D**).

Tigecycline Slows Down Protein Translation but Boosts Ribosome Synthesis

In line with the known mode of action of tigecycline, where its interaction with the 16S rRNA directly blocks the aminoacyl-tRNA from entering the ribosomal A site (Jenner et al., 2013), the RNA-Seq data shows clear evidence of ribosomal stalling after translational initiation. Fourteen genes encoding aminoacyl-tRNA synthetases (**Figure 1A**) and 27 genes responsible for amino acid synthesis (**Figure 1C**) were transcriptionally down regulated, possibly because of a reduced turnover of aminoacyl-tRNA/tRNA, and a reduced rate of amino acid incorporation into polypeptide chains. Correspondingly, transcript abundance was also decreased for two EF-Tu (*tuf*) genes and the EF-P encoding gene (EF-P, *efp*), which participates in peptidyl transferase activity

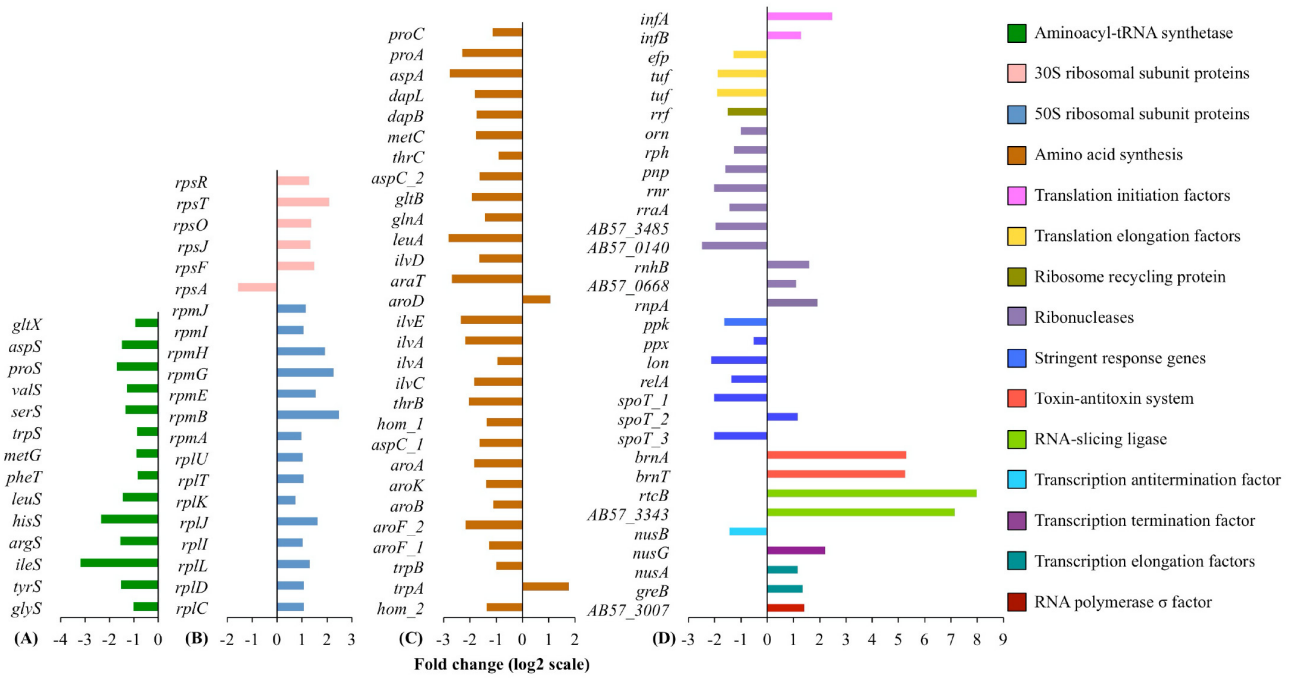

FIGURE 1 | (A) Aminoacyl-tRNA synthetases, **(B)** ribosomal proteins, **(C)** amino acid synthesis, and **(D)** others. Differential expression of genes involved in, or affecting, protein translation. For each functional group or cellular pathway, only differentially transcribed genes with absolute fold changes >1 ($\log_2$ scale) and padj < 0.01 are shown. The bar charts are grouped by colors according to the biological functions of the genes assigned, with the color scheme on the right. Full information on gene function annotations is given in **Supplementary Data**.

on the ribosomal P (peptidyl) site (**Figure 1D**). Transcription of the ribosome recycling factor (RRF) encoding gene *frr* was also decreased in response to tigecycline (**Figure 1D**). RRF is required for 70S ribosome separation at the end of each peptide translation cycle and ribosomal subunit recycling for the subsequent round of translation (Wilson, 2009). These observations suggest a slower protein translation rate, and are consistent with the known initial antibacterial action of tigecycline in protein translation perturbation (Jenner et al., 2013). Decreased transcript levels of genes involved in amino acid synthesis and tRNA aminoacylation were also reported in *Streptococcus pneumoniae* when treated with translational inhibitors including tetracycline, chloramphenicol, erythromycin and puromycin (Ng et al., 2002). Our RNA-seq data also showed decreased transcriptional levels of twelve genes encoding proteases, potentially indicative of a slower protein degradation rate following tigecycline treatment.

The transcription of genes for sixteen 50S and five 30S ribosomal proteins were increased (**Figure 1B**), consistent with previous findings in *S. pneumoniae*, *Escherichia coli*, and *Haemophilus influenzae* following treatment with translational inhibitors including tetracycline and transcriptomic or proteomic measurement of expression changes (VanBogelen and Neidhardt, 1990; Evers et al., 2001; Ng et al., 2002). The *H. influenzae* study demonstrated that translational inhibitors increased the total RNA synthesis rate, and hence the corresponding rate of rRNA synthesis, as this is the major RNA species in the cell (Evers et al., 2001). rRNA synthesis is the rate-limiting step in ribosome synthesis and assembly (Maaløe and Kjeldgaard, 1966). Our study used rRNA-depleted samples, so we could not directly verify whether the rRNAs were increased in abundance upon tigecycline treatment. However, transcription was observed to increase for two translation initiation factors IF-1 (*infA*) and IF-2 (*infB*), required for the assembly of ribosome subunits to start a new protein translation cycle, consistent with the *A. baumannii* cellular response including synthesis of ribosomal proteins and rRNA and a greater need for ribosomal assembly.

The transcriptional changes in genes responsible for several cellular activities involved with guanosine tetraphosphate and guanosine pentaphosphate (p)ppGpp further support the notion that, like other translational inhibitor antibiotics, tigecycline could boost rRNA and ribosome biosynthesis in *A. baumannii*. (p)ppGpp is a negative effector of the *rrnB P1* promoter of rRNA operons (Murray et al., 2003). In adapting to amino acid starvation, overproduction of (p)ppGpp inhibits ribosome synthesis directly and mediates ribosomal protein degradation through Lon protease, via the stringent response (Kuroda et al., 2001; Wendrich et al., 2002). Upon tigecycline treatment, we observed decreased expression levels of various genes responsible for (p)ppGpp synthesis and degradation (**Figure 1D**, stringent response genes). These include *relA*, encoding a (p)ppGpp synthetase, *spoT*, encoding a synthetase and hydrolase of (p)ppGpp, and *lon,* encoding Lon protease. In addition, we observed decreased expression of genes impacting cellular

levels of polyphosphate (polyP) – a known cofactor of Lon-mediated free ribosomal protein degradation (Kuroda et al., 2001) – including *ppk*, encoding polyphosphate kinase, and *ppx*, encoding exopolyphosphatase. These results, together with the observation of reduced (p)ppGpp cellular level in translational inhibitor antibiotics stressed bacterial cells (Evers et al., 2001), suggest that tigecycline could also induce the reduction of (p)ppGpp production in *A. baumannii*. Such reduced (p)ppGpp production would be expected to stimulate rRNA synthesis and inhibit Lon-mediated ribosomal protein degradation.

Central Metabolism and Cell Division

Bacteriostatic antibiotics capable of inducing translation perturbation have been reported to have profound downstream consequences on suppressing bacterial metabolism, including the accumulation of metabolites from central metabolic and cellular respiration pathways (Lin et al., 2014; Lobritz et al., 2015). Our RNA-Seq data showed consistent tigecycline-induced reductions in gene expression for enzymes in the tricarboxylic acid (TCA) cycle and cellular respiration (**Figures 2A,B**). Transcription of the genes responsible for cell wall metabolism and cell division was also down regulated by tigecycline (**Figure 2C**). The effect of tigecycline on transcription of genes involved in translation perturbation, RNA degradation, TCA cycle and respiration, cell division and cell-wall synthesis, reflect the reduction in cellular growth rate in *A. baumannii* 6772166, potentially linked to tigecycline's bacteriostatic effect (Cocozaki et al., 2016).

Genes With Highest Transcriptional Increases Are Involved in RNA Metabolism

A plasmid-borne toxin-antitoxin (TA) system, homologous to a chromosomal TA system *brnT/brnA* first characterized in *Brucella abortus* (Heaton et al., 2012), showed 39-fold increased expression in response to tigecycline (**Figure 1D**). BrnT is a type II ribonuclease toxin which when co-expressed with the antitoxin protein BrnA forms a tetramer $BrnT_2$-$BrnA_2$ that neutralizes BrnT toxicity and functions as a strong autorepressor of its own operon (Heaton et al., 2012). However, a decrease in the cellular level of BrnA can de-repress expression of the TA system and lead to increased levels of BrnT, which in turn inhibits cell growth through RNA degradation, thus interfering with protein translation (Heaton et al., 2012). Chloramphenicol was shown to stimulate the overexpression of *brnT/brnA* in *B. abortus* (Heaton et al., 2012). A potential mechanism behind the increased transcription of this TA system in our current study is that the antitoxin is less stable and upon translational inhibition induced by either chloramphenicol or tigecycline, the antitoxin will become inactive, the TA complex will dissociate and thus transcription of the TA operon will be derepressed.

The two genes with the highest fold expression increase (252-fold) were *rtcB*, encoding a tRNA repair enzyme, and a hypothetical gene AB57_3343 directly downstream of *rtcB* (**Figure 1D**). *rtcB* was initially characterized in *E. coli* as a stand-alone ligase involved in healing and sealing broken tRNA-like stem-loop structures *in vitro*, and was later demonstrated to catalyze tRNA and mRNA repair in yeast *in vivo* (Tanaka and Shuman, 2011; Tanaka et al., 2011). We speculate that *rtcB* overexpression could be linked to *brnT/brnA* overexpression, as it may play a role evading the potential programmed RNA breakage mediated by this TA system, and RtcB could potentially serve as an indirect tigecycline induced stress-response determinant. Another possibility is that blockage of tRNAs to the ribosome caused by tigecycline leads to a cellular response in tRNA repair. *rtcB* was also significantly up-regulated in the previous study of *A. baumannii* MDR-ZJ06 transcriptomic response to tigecycline (Hua et al., 2014). In either case, given the very strong upregulation of transcription of this gene, RtcB could potentially serve as an indirect tigecycline induced stress-response determinant.

Impacts on DNA Repair and Horizontal Gene Transfer

Transcriptomic data presented here suggests that tigecycline may impact DNA repair and DNA competence and mobility. For instance, the transcription-coupled nucleotide excision repair (TCR) genes *mfd*, *uvrA*, *uvrB*, and *uvrC* all showed decreased transcription (**Figure 3**). Mfd and UvrA are important in bacterial mutagenesis and evolution of antimicrobial resistance during host infection where DNA replication in the bacterial cells is low while transcription is still active (Ragheb et al., 2019). Furthermore, the expression of the transcription EF gene *greB* that interferes TCR activities was increased, and the expression of (p)ppGpp synthesis genes were decreased, which are required for efficient TCR activities (**Figure 1D**; Epshtein et al., 2014; Kamarthapu et al., 2016). These findings, that tigecycline exposure diminishes TCR activities via mechanisms including (p)ppGpp reduction, collectively suggest that such exposure can result in an increased TCR-related mutation rate.

Increased expression was observed for *mutS,* encoding the DNA mismatch repair (MMR) enzyme, and two other putative DNA repair genes AB57_2357 and AB57_1251 (**Figure 3**). Tigecycline has been reported to generate tigecycline-resistant hypermutator strains of *A. baumannii* either *in vitro* (in a bioreactor) or *in vivo* (patient with tigecycline therapy), linked to insertion sequence (IS)-mediated inactivation of *mutS* (Hammerstrom et al., 2015). Speculatively, the emergence of tigecycline resistant strains linked to insertional disruption of the *mutS* gene, may reflect that *mutS* is the principle mismatch repair system induced by tigecycline at sub-inhibitory concentrations (**Figure 3**); and thus inactivation of *mutS* would substantially enhance mutation rates.

Tigecycline treatment increased expression of various genes associated with DNA mobility, including twenty-five putative transposase genes and four homologs of the DNA mobilization gene *bmgB* (**Supplementary Figure 4**), which could be a potential factor in the rapid emergence of IS-inactivated *mutS* mutants in *A. baumannii* previously reported (Hammerstrom et al., 2015). Genes involved in DNA uptake were also induced by tigecycline treatment including the cell competence genes *comM*, *cinA*, and *comEA*, and conjugal transfer protein *traR* genes. These changes in expression of DNA competence and mobilization

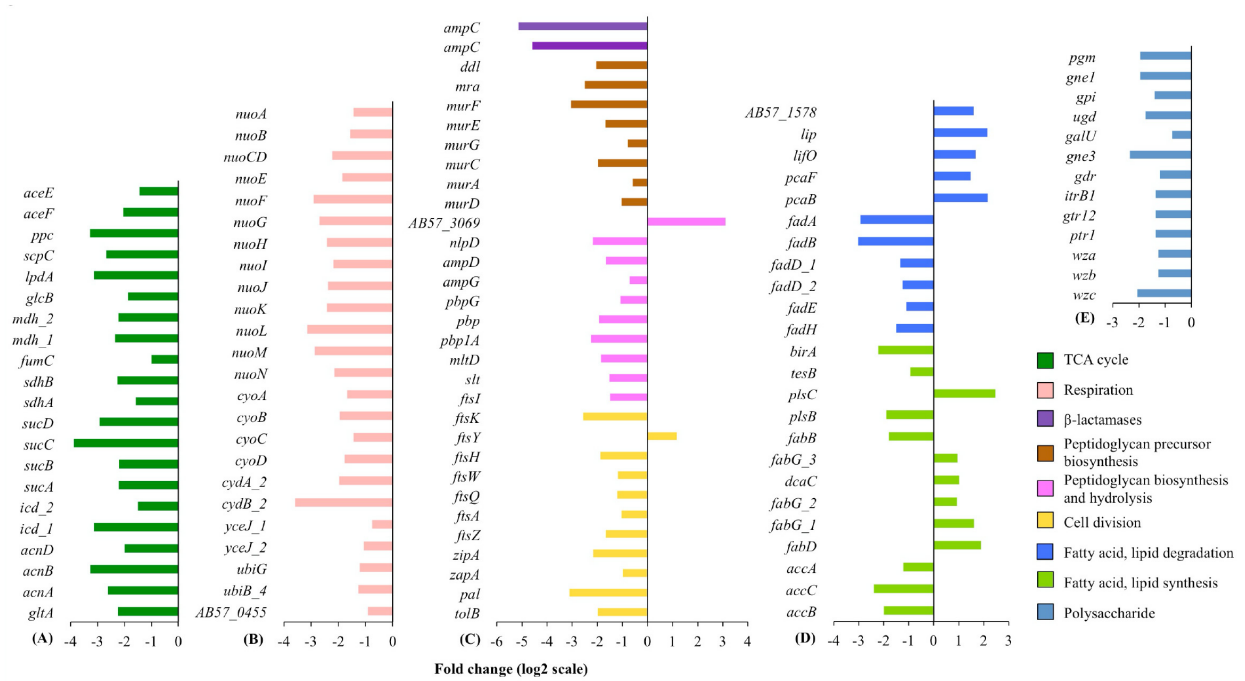

FIGURE 2 | (A) TCA cycle, **(B)** respiration, **(C)** cell wall and cell division, **(D)** fatty acid and lipid, and **(E)** polysaccharide. Differential transcription of genes in key metabolic pathways. For each cellular pathway, only differentially transcribed genes with absolute fold changes >1 ($\log_2$ scale) and padj < 0.01 are shown. The bar charts are grouped by colors according to the biological functions of the genes assigned, with the color scheme on under panel **(E)**. Full information on gene function annotations is given in **Supplementary Data**.

genes suggests that tigecycline may affect horizontal gene transfer (HGT) rates. Consistent with this hypothesis, an operon encoding the type VI secretion system (T6SS) showed increased expression following tigecycline treatment (**Figure 3**). The T6SS in *Vibrio cholerae* was shown to be up-regulated through competence induction, releasing the DNA of the surrounding non-immune cells through deliberate killing to make the DNA available for uptake (Borgeaud et al., 2015). Expression of a lytic transglycosylase gene, proposed to play a role in creating space within the peptidoglycan sacculus for the insertion of cell-envelope spanning structures, such as T6SS, was also induced by tigecycline (Scheurwater et al., 2008).

Tigecycline and β-Lactams

The most highly down-regulated genes in this study were two *ampC*β-lactamase-encoding genes (**Figure 2C**), whose transcription was reduced by 35-fold and 24-fold. AmpC β-lactamases are active against five classes of β-lactams (Jacoby, 2009). β-lactams can induce a toxic malfunctioning of the cycle of peptidoglycan synthesis, hydrolysis and recycling, and induction of β-lactamase genes typically correlates with peptidoglycan recycling (Jacoby, 2009; Li et al., 2016b). Interestingly, tigecycline also induced decreased transcription of four DD-carboxypeptidase-encoding genes (**Supplementary Data**). Speculatively, reduced expression of peptidoglycan biosynthesis genes (**Figure 2C**) might lead to, or be accompanied by, reduced production of N-acetylglucosamine moieties, which positively affect β-lactamase gene expression (Jacobs et al., 1994, 1997). This supports an intriguing possibility that tigecycline and β-lactams could be used synergistically in combination therapy, as has been hypothesized previously (Tumbarello et al., 2012). To investigate the potential synergy between tigecycline and β-lactams, we conducted checkerboard assays on *A. baumannii* 6772166 with tigecycline and five AmpC β-lactam substrates, respectively, including piperacillin, carbenicillin, ceftriaxone, cefotaxime and cefuroxime (Garcia, 2010). While the fractional inhibitory concentration indexes indicated no clinically significant synergy, inclusion of tigecycline at 2.5 μg/ml resulted in a four to eight-fold decrease in the MIC of these five β-lactams for *A. baumannii* 6772166 (**Table 1**). Bactericidal antibiotics, such as β-lactams, induce reactive oxygen species (ROS) dependent killing (Kohanski et al., 2007); whereas tigecycline at a subinhibitory concentration causes the downregulation of TCA cycle and respiration, both of which would likely limit ROS generation (Lobritz et al., 2015). This may explain why we only observed low synergy between tigecycline and the β-lactams, despite tigecycline-induced *ampC* downregulation.

Drug Efflux and Uptake

A common mechanism for resistance to tetracycline antibiotics involves reducing drug intracellular concentration via efflux pumps and/or down regulation of the outer membrane proteins (OMP) responsible for drug uptake (Piddock, 2006). The multidrug efflux pumps previously characterized in *A. baumannii*

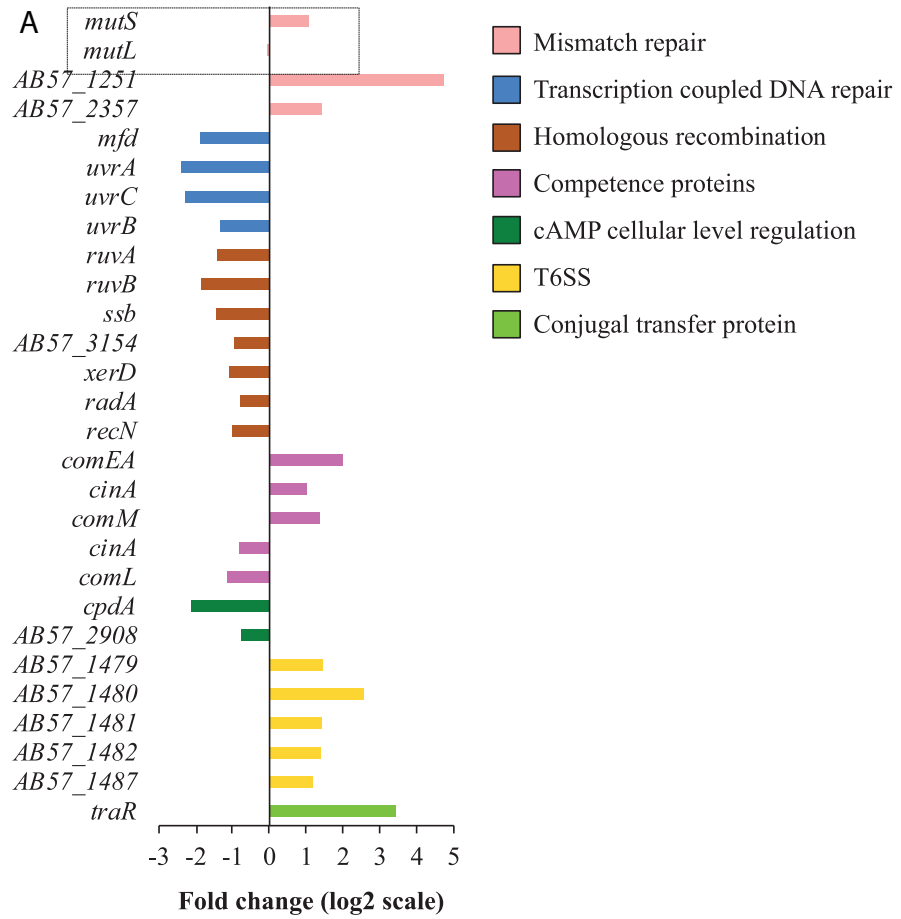

FIGURE 3 | Tigecycline affects transcription of genes involved in DNA repair and HGT. **(A)** For each functional group, only differentially transcribed genes with absolute fold changes >1 (log_2 scale) and padj < 0.01 are shown. The bar charts are grouped by colors according to the biological functions of the genes assigned, with the color scheme on the right. Full information on gene function annotations is given in **Supplementary Data**.

associated with tigecycline resistance are AdeABC and AdeIJK (Peleg et al., 2007; Damier-Piolle et al., 2008). The AdeABC-mediated tigecycline resistant *A. baumannii* strains reported previously showed mutations in either *adeR* or *adeS*, leading to its constitutive overexpression and hence tigecycline resistance (Peleg et al., 2007; Sun et al., 2014). However, in our RNA-Seq data *adeA* and *adeC* were down-regulated by tigecycline by more than two-fold, and the *adeIJK* operon was not differentially transcribed (**Figure 4A**). Although conferring resistance to tigecycline, the expression of *adeABC* and *adeIJK* are not positively responsive to the drug. This probably explains why constitutive overexpression of these two RND systems is commonly observed in tigecycline resistant *A. baumannii* strains.

TABLE 1 | Results of checkerboard assay between tigecycline and β-lactams[a]

	β-lactam MIC (mg/ml)[b]		Tigecycline MIC (μg/ml)[b]		FICI[c]
	Alone	With tigecycline	Alone	With β-lactam	
Piperacillin	2	0.25	5	2.5	0.625
Carbenicillin	8	2	5	2.5	0.75
Ceftriaxone	2	0.5	5	2.5	0.625
Cefotaxime	0.5	0.125	5	2.5	0.75
Cefuroxime	2	0.5	5	2.5	0.75

[a]For each of the five checkerboard assays, two biological replicates were performed. [b]The wells picked for FICI calculation were with lowest growth inhibitory concentration of β-lactams. This was to show that tigecycline at subinhibitory concentration increased A. baumannii susceptibility to β-lactams. The wells with similar FICI but of lower tigecycline inhibitory concentrations were not shown. [c]The defined clinically significant synergy is of FICI ≤ 0.5.(Odds, 2003).

Two genes encoding characterized drug efflux pumps (*tetA* and *craA*) and ten genes encoding hypothetical proteins with predicted transmembrane helices were found to be transcriptionally responsive to tigecycline (**Figure 3A**). The tetracycline exporter gene *tetA* and its transcriptional repressor gene *tetR* were both upregulated (**Figure 3A**), which is consistent with previous observations that tigecycline could induce *tetA* overexpression in *E. coli* but *tetA* does not confer tigecycline resistance (Hirata et al., 2004). CraA is a MDR efflux pump that transports chloramphenicol (Roca et al., 2009) and other antimicrobials (Li et al., 2016a). To further investigate

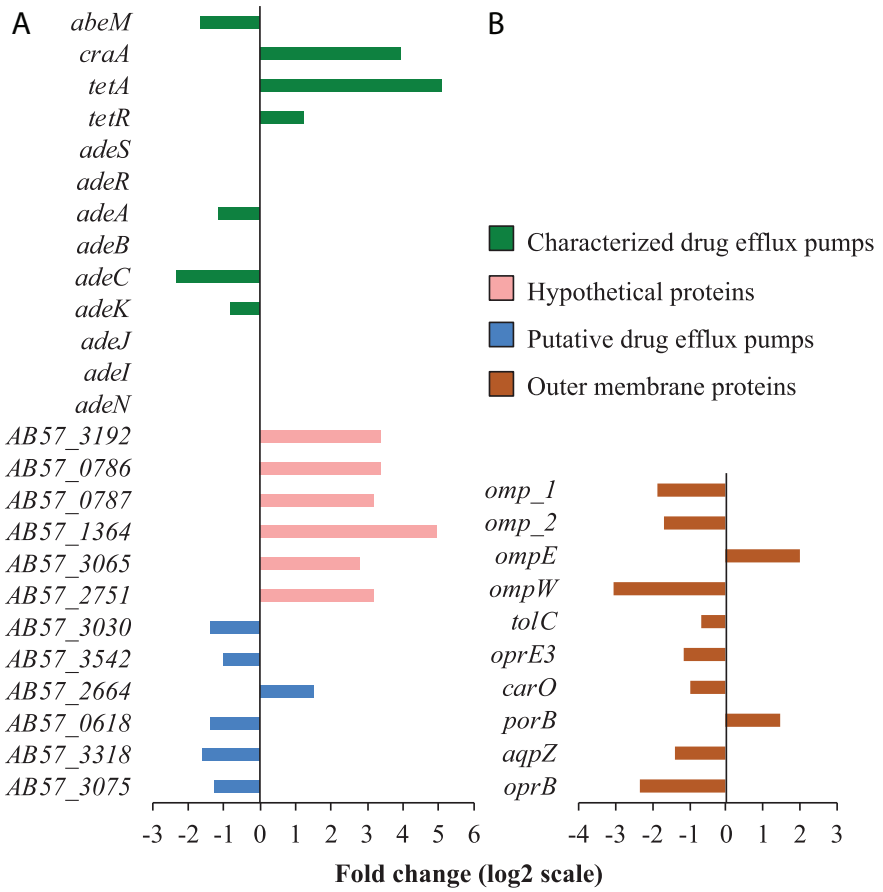

FIGURE 4 | Tigecycline impact on the transcription of genes involved in membrane permeability and transport. **(A)** The characterized drug efflux pumps presented here are either the ones differentially regulated, or the ones known in conferring tigecycline resistance. The hypothetical proteins are responsive to tigecycline and predicted to have transmembrane helixes. **(B)** Only the differentially regulated OMPs are presented. All the genes on the panel **(A,B)** have absolute fold change more than 1 (log_2 scale) with statistical significance (padj < 0.01). Full information on gene function annotations is given in **Supplementary Data**.

whether CraA and the ten hypothetical membrane proteins do confer tigecycline resistance, these genes were heterologously overexpressed in *E. coli*. However, no significant resistance phenotype was observed for any of these clones, suggesting that although transcriptionally responsive to tigecycline, they do not mediate tigecycline resistance, or at least not at an easily detectable level.

Tigecycline treatment also led to decreased transcription of several OMP genes (**Figure 3B**) and genes involved in capsular polysaccharide biosynthesis and export (**Figure 2E**). These changes might affect bacterial outer membrane permeability and thus tigecycline uptake.

CONCLUSION

In this study, we used RNA-Seq to show that exposure to a sub-inhibitory concentration of tigecycline in *A. baumannii* affected transcription of genes involved in protein translation as expected, but also resulted in significant gene transcriptional changes associated with RNA metabolism, DNA mismatch repair, HGT and genetic element mobilization. It also reduced the transcriptional levels of genes involved in the biogenesis of key cellular components, including the cell membrane, peptidoglycan, respiration chain and TCA cycle. Tigecycline was found to increase the susceptibility of *A. baumannii* to five β-lactams, which is hypothesized to be correlated with the tigecycline-induced reduction of peptidoglycan biogenesis accompanied by the downregulation of two β-lactamase encoding genes.

The core tigecycline cellular response appears to be mainly coordinated through down-regulation of (p)ppGpp, which modulates ribosome production to counteract translational inhibition effect of tigecycline, and impairs TCR-related DNA mismatch repair. Furthermore, tigecycline may increase rates of IS element transposition. These effects collectively may increase the rate of emergence of tigecycline-resistant mutants. One question arising from this study is whether the other translation-inhibiting antibiotics affect DNA repair pathways in the same way as seen here for tigecycline. Ribosome stalling and (p)ppGpp reduction are common translational inhibitor-induced responses in various bacterial species, therefore the related physiological

changes, such as TCR-related DNA mismatch repair, are likely the core tigecycline transcriptomic signatures shared across many bacteria. Given the known variation in genotypes and phenotypes across characterized *A. baumannii* isolates, it is likely there will be some degree of strain to strain variation in transcriptional responses to tigecycline.

Tigecycline, a treatment of last resort for infections by opportunistic, MDR pathogens, remains an important clinical resource. Here we show that *A. baumannii* mounts a more complex suite of cellular responses to such treatment than previously appreciated and reveal why hypermutation can occur during treatment.

MATERIALS AND METHODS

Bacterial Strains and Genome Sequences

Acinetobacter baumannii 6772166, kindly gifted by Dr. Melisa Brown (Eijkelkamp et al., 2011), with a tigecycline MIC between 2.5 and 5 μg/ml, was used for this transcriptomic study. The genomic DNA (gDNA) was sequenced (GEO accession no: GSE131451) and shown to share high identity to the genome sequence of AB0057 (**Supplementary Figure 1**). The plasmid and chromosome sequences of *A. baumannii* AB0057 were used as the reference sequence for the subsequent RNA-seq read alignment and statistical analyses. *E. coli* strain DH5α (α-select chemically competent cells, Bioline Australia) was used for heterologous overexpression and functional characterization for the putative transport systems.

Tigecycline Transcriptomic Analysis by RNA-Seq

Acinetobacter baumannii 6772166 Muller-Hinton (Oxoid, United States) broth cultures at OD_{600} 0.6 with and without 2.5 μg/ml tigecycline (Sigma-Aldrich, United States) treatment were grown for 30 min at 37°C with shaking. Three biological replicates were prepared. The total RNA of each sample was extracted using the miRNeasy Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. rRNA was depleted by using the Ribo-Zero Magnetic kit (bacteria) (Illumina, Inc., United States), and converted to cDNA which was sequenced via Illumina HiSeq2500 at Ramaciotti Center for Genomics. Nearly 75 million unique 101 bp reads were obtained from each RNA sample representing more than 310-fold genome coverage. The sequence reads from the 6 RNA-Seq samples (GEO accession no: GSE131451) were aligned against the *A. baumannii* AB0057 genome and transcriptional coverage for each gene was determined using EDGE-pro (Estimated Degree of Gene Expression in Prokaryotic Genomes) (Magoc et al., 2013). More than 99% of the RNA-Seq reads of each sample were mapped to AB0057 genome sequence. Genes with significantly different transcription between the control and experimental samples were identified using the DEseq R package (**Supplementary Data**). Gene differential expression was visualized by metabolic cellular view in Biocyc (Caspi et al., 2016; Karp et al., 2019).

Functional Validation of Membrane Transporters Through Heterologous Overexpression

Eight genes, including two efflux pumps and six hypothetical proteins with at least three transmembrane helices predicted by the TMHMM Server v.2.0., were cloned into pTTQ18 and overexpressed in *E. coli* DH5α as previously described (Hassan et al., 2011). The MIC of each *E. coli* clone was determined through two-fold serial broth dilution in MH broth according to the guidelines of CLSI (Clinical and Laboratory Standards Institute), with two biological replicates.

Tigecycline and β-Lactam Antibiotics Synergism Validation Through Checkerboard Assay

The synergism between tigecycline and five β-lactam antibiotics, namely piperacillin, carbenicillin, ceftriaxone, cefotaxime and cefuroxime (all from Sigma-Aldrich, United States) were determined through broth microdilution checkerboard assay following previously developed methodology (Garcia, 2010). Two independent biological repeats were performed for each combination set.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

IP, KH, and LL conceptualized the work. LL conducted all experiments with assistance from AP and VN. LL performed transcriptomic the data analyses with significant assistance and contribution from ST. LL, IP, KH, AC, and ST worte the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.565438/full#supplementary-material

Supplementary Figure 1 | Mauve visualization of genomic identity between *A. baumannii* AB0057 and *A. baumannii* 6772166. The horizontal track on the top represents the chromosome sequence of *A. baumannii* AB0057, and the one on the bottom represents the rearranged contig sequences of *A. baumannii* 6772166 with AB0057 as the reference. The long red vertical lines on the bottom track represent the boundaries of each contig. A colored similarity plot is shown for each genome, the height of which is proportional to the level of sequence identity in that region.

Supplementary Figure 2 | A global view of differential gene transcription induced by tigecycline. The circular map was based on the reference genome *A. baumannii* AB0057. From the outermost circle in, the circle 1 shows the ORFs on the plus strand of AB0057 chromosome, the circle 2 the ORFs on the minus strand, the circle 3 shows the degree of differential transcription (increased or decreased) for each gene, and the circle 4 shows the coordinates of the chromosome. The ORFs are color coded according to their respective COG category, and with the color scheme on the right. This map was constructed using a web-based tool Circular Visualization for Microbial Genomes (Overmars et al., 2015).

Supplementary Figure 3 | Differential transcription of other membrane transporter encoding genes. Only the differently regulated membrane transporter genes are presented, and their absolute fold changes are more than 1 ($\log_2$ scale) with padj < 0.01. The genes on panels **(A)** and **(B)** are color grouped based on the putative substrate specificities. The genes on panel **(C)** are grouped based on transporter families, as they have no substrate predicted. Full information on gene function annotations is given in supplementary data.

Supplementary Figure 4 | Increased transcription of transposase genes and DNA mobilization genes. For each functional group, only differentially transcribed genes with absolute fold changes >1 ($\log_2$ scale) with padj < 0.01 are shown. The bar charts are grouped by colors according to the biological functions of the genes assigned, with the color scheme on the right. Full information on gene function annotations is given in supplementary data.

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Orally Administered Antibiotics Vancomycin and Ampicillin Cause Cognitive Impairment With Gut Dysbiosis in Mice With Transient Global Forebrain Ischemia

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Gut microbiota is closely associated with the occurrence of neuropsychiatric disorders. Antibiotics are frequently used to prevent pathogen infection in patients with brain ischemia. To understand the impact of prophylactic antibiotic treatment for patients with brain ischemia, we examined the effects of orally administered vancomycin and ampicillin on cognitive function and gut microbiota composition in mice with transient global forebrain ischemia (tIsc). tIsc operation and orally gavaged vancomycin mildly and moderately caused cognitive impairment, respectively. However, the exposure of mice with tIsc to vancomycin or ampicillin severely impaired cognitive function in the Y-maze, novel object recognition, and Banes maze tasks. Furthermore, their treatments induced NF-κB activation as well as active microglia ($NF\text{-}\kappa B^{+}/Iba1^{+}$ and $LPS^{+}/Iba1^{+}$ cells) and apoptotic (caspase $3^{+}/NeuN^{+}$) cell population in the hippocampus, whereas the brain-derived neurotrophic factor $(BDNF)^{+}/NeuN^{+}$ cell populations decreased. These treatments also caused colitis and gut dysbiosis. They increased the population of Proteobacteria including *Enterobacter xiangfangenesis.* Orally delivered fecal transplantation of vancomycin-treated mice with or without tIsc and oral gavage of *Enterobacter xiangfangenesis* also significantly deteriorated the cognitive impairment and colitis in transplanted mice with tIsc. These findings suggest that oral administration of antibiotics can deteriorate cognitive impairment with gut dysbiosis in patients with brain ischemia.

Keywords: antibacterials, brain ischemia, memory, gut dysbiosis, fecal transplantation, *Enterobacter xiangfangenesis*

INTRODUCTION

Brain ischemia occurs when there is an insufficient amount of blood flow to the brain (Clemens et al., 1997; Bramlett and Dietrich, 2004). This deprives brain tissue of oxygen and nutrients, resulting in brain cell death, cerebral infarction, or ischemic stroke. Brain inflammation occurs in the damaged brain tissue following the infiltration of immune cells, which secrete proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in ischemic brain injury, and this leads to the impairment of brain functions such as cognition (Kawabori and Yenari, 2015; Anttila et al., 2017). Microbial infection deteriorates the prognosis of ischemic stroke (Fugate et al., 2014). The prophylactic administration of antibiotics is recommended to reduce the risk of pathogen infections in patients with ischemic brain injury (Enzler et al., 2011; Xi et al., 2017). Therefore, several studies examined the impact of antibiotic treatment on the outcomes in hosts with ischemic brain injury. For example, Benakis et al. (2016) reported that orally administered amoxicillin/clavulanic acid reduces ischemic brain injury in mice by regulating gut microbiota composition, while Dong et al. (2017) reported that orally prophylactic treatment with an antibiotic cocktail consisting of chlortetracycline, penicillin VK, and vancomycin could not reduce microbial infection, and Winek et al. (2016) reported that the depletion of gut microbiota by oral prophylactic treatment with an antibiotic cocktail consisting of ampicillin, vancomycin, ciprofloxacin, imipenem, and metronidazole deteriorates the outcome after murine stroke. Singh et al. (2016) reported that fecal microbiota transplantation of healthy mice into middle cerebral artery (MCA) occlusion-operated mice alleviated the stroke outcome.

Recent studies have indicated that the alteration of gut microbiota by gut environmental factors such as orally administered antibiotics and stressors causes the occurrence of psychiatric disorders with colitis (Lundberg et al., 2016; Ceylani et al., 2018; Cussotto et al., 2018; Jang et al., 2018a). For example, Ceylani et al. (2018) reported that oral administration of ampicillin and cefoperazone caused anxiety and suppressed BDNF expression in mice. Jang et al. (2018a) reported that oral gavage of ampicillin and fecal transplantation of ampicillin-treated mice significantly increased anxiety-like behaviors and caused gut dysbiosis in the transplanted conventional animals. The induction of gut dysbiosis by 2,4,6-trinitrobenzenesulfonic acid (TNBS), a colitis inducer, or immobilization stress increases the lipopolysaccharide (LPS) level in the feces and blood, and inflammation in the colon and hippocampus and suppresses BDNF expression in the hippocampus, resulting in the occurrence of psychiatric disorders (Cani et al., 2008; Deng et al., 2014; Lee et al., 2017; Jang et al., 2018b, c; Rosenzweig et al., 2004). Soto et al. (2018) reported that oral administration of vancomycin reduced brain-derived neurotrophic factor (BDNF) expression and anxiety/depression in mice. These reports suggest that gut dysbiosis is closely associated with the occurrence of psychiatric disorders. Nevertheless, the interplay between antibiotics-induced gut dysbiosis and the occurrence and development of psychiatric disorders in patients with brain ischemia remains unclear.

Therefore, to understand the effects of the antibiotics that are used to prevent pathogen infection on the occurrence of cognitive impairment in patients with brain ischemia, we examined the effects of the orally administered antibiotics vancomycin and ampicillin on cognitive function, gut microbiota composition, and gut and brain inflammation in mice with transient global forebrain ischemia (tIsc).

RESULTS

Orally Administered Vancomycin and Ampicillin Caused Cognitive Impairment in Mice With tIsc

First, to understand the effects of the antibiotics used in patients with brain ischemia on the occurrence of cognitive impairment, we induced transient global forebrain ischemia in mice, orally administered ampicillin or vancomycin, and examined cognitive function (**Figure 1A**). The induction of tIsc itself caused mild cognitive impairment in the Y-maze, novel object recognition maze, and Barnes maze tasks compared to those of control and sham mice. Exposure of mice with tIsc to orally administered vancomycin or ampicillin significantly deteriorated cognitive impairment compared to tIsc mice not treated with antibiotics (**Figures 1B–D** and **Supplementary Figure S1**). However, antibiotic treatments did not significantly affect the distance moved in the behavioral tests [the means of arm entries in the Y-maze task and total (target and error) hole visit number in the Barnes maze task] compared to those of the control group treated with the vehicle. Orally administered vancomycin worsened the cognitive function in the Barnes maze task more severely than the orally administered ampicillin. Oral administration of vancomycin or ampicillin increased TNF-α and IL-1β levels (**Figures 1E,F**). These treatments also increased the $Iba1^+$ cell population in the hippocampus of mice with tIsc more strongly than in tIsc mice not treated with antibiotics. In particular, treatment with vancomycin or ampicillin increased the $Iba1^+$ cell population in the CA1 region of the hippocampus more than in the CA2 and CA3 regions (**Figures 1G,H** and **Supplementary Figure S2**). Furthermore, these antibiotic treatments increased the $LPS^+/Iba1^+$ and $NF\text{-}\kappa B^+/Iba1^+$ cell (activated microglia) populations in the CA1 region of the hippocampus of mice with tIsc more strongly than in tIsc mice not treated orally with vancomycin or ampicillin. Furthermore, treatment with antibiotics increased the caspase $3^+/NeuN^+$ (apoptotic neuron) cell population in the CA1 region of the hippocampus, while the $BDNF^+/NeuN^+$ cell population was suppressed (**Figures 1I,J** and **Supplementary Figure S3**). The induction of tIsc increased the blood TNF-α level in mice somewhat, but not significantly, compared to those of control and sham mice. However, orally administered vancomycin or ampicillin markedly increased blood LPS and TNF-α levels in mice with tIsc (**Figures 1K,L**).

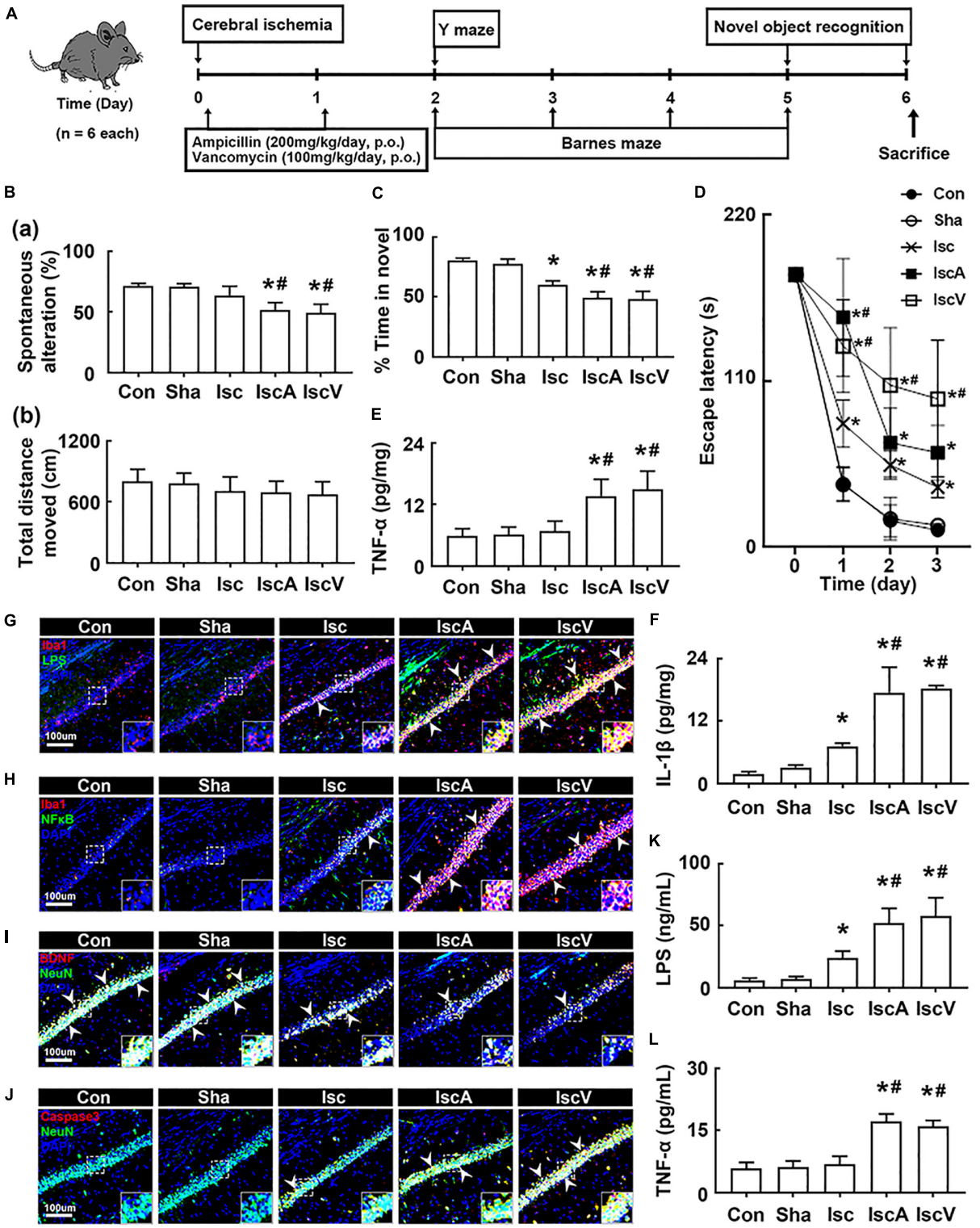

FIGURE 1 | Vancomycin and ampicillin caused the cognitive impairment in mice with and without tIsc. **(A)** Experimental protocol. Effects on the cognitive function, assessed by the Y-maze [**(B)** a, spontaneous alteration; b, moved distance], novel object recognition **(C)**, and Banes maze tasks **(D)**. Effects on TNF-α **(E)** and IL-1β expression **(F)** in the hippocampus, assessed by ELISA. Effects on $LPS^{+}/Iba1^{+}$ **(G)**, $NF\text{-}\kappa B^{+}/Iba1^{+}$ **(H)**, $BDNF^{+}/NeuN^{+}$ **(I)**, and caspase $3^{+}/NeuN^{+}$ cell **(J)** populations in the hippocampus, assessed by a confocal microscope. Effects on LPS **(K)** and TNF-α levels **(L)** in the blood. Con, Sha, Isc, IscA, and IscV in the *x*-axis of figures indicate groups treated with vehicle in normal control mice, vehicle in sham mice, vehicle in mice with tIsc, ampicillin in mice with tIsc, and vancomycin in mice with tIsc, respectively. All data were expressed as mean ± SD ($n = 6$). $^{*}p < 0.05$ vs. Sha group. $^{\#}p < 0.05$ vs. Isc group.

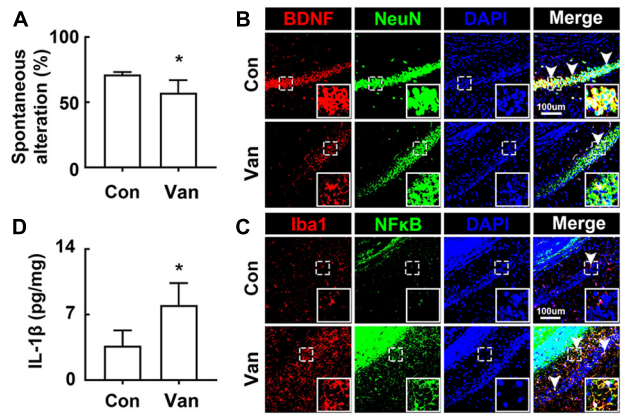

FIGURE 2 | Vancomycin caused the cognitive impairment in mice. **(A)** Effects on the cognitive function, assessed by the Y-maze task. Effects on BDNF$^+$/NeuN$^+$ **(B)** and NF-κB$^+$/Iba1$^+$ cell populations **(C)** and IL-1β expression **(D)** in the hippocampus. Con and Van in the *x*-axis of figures indicate groups treated with vehicle and vancomycin (150 mg/kg/day) for 2 days in normal control mice, respectively. All data were expressed as mean ± SD (n = 6). *p < 0.05 vs. control group.

Next, we examined the effect of orally administered vancomycin in control mice. Oral gavage of vancomycin significantly caused cognitive impairment in the Y-maze task and increased the IL-1β level in the hippocampus, as previously reported in mice treated with ampicillin (Jang et al., 2018a; **Figures 2A,D**). Furthermore, vancomycin treatment also increased the BDNF$^+$/NeuN$^+$ cell population, while the NF-κB$^+$/Iba1$^+$ cell population decreased (**Figures 2B,C**).

Orally Administered Vancomycin and Ampicillin Deteriorated tIsc-Induced Gut Inflammation in Mice

To understand whether the occurrence of cognitive impairment by antibiotics was associated with gut dysbiosis, we examined the effects of orally administered ampicillin and vancomycin on gut microbiota composition and colitis in mice with tIsc. The induction of tIsc did not significantly affect gut microbiota composition compared to those of control and sham mice. However, oral administration of vancomycin or ampicillin in mice with tIsc significantly reduced α-diversity and shifted β-diversity in the gut microbiota composition (**Figures 3A,D**). Oral administration of vancomycin significantly increased the Proteobacteria population and decreased the Verrucomicrobia population (**Figures 3B,E**). Orally administered ampicillin also increased the Proteobacteria population, as previously reported in normal control mice (Jang et al., 2018a). Furthermore, orally administered vancomycin and ampicillin increased the population of Enterobacteriaceae including *Enterbacter* sp. in mice with tIsc (**Figure 3C**, **Supplementary Figure S4A**, and **Supplementary Tables S1**, **S2**). Therefore, to confirm whether these antibiotic treatments could increase the Proteobacteria population in mice with tIsc, we cultured the fecal bacteria of orally vancomycin-treated mice with tIsc in a DHL agar plate, which is a selective medium for Enterobacteriaceae (**Figure 3E**). Oral administration of vancomycin significantly increased the population of *Enterobacter xiangfangenesis,* which belongs to the Proteobacteria family, in the DHL agar plate compared to those of control, sham, and tIsc mice. tIsc operation weakly increased fecal LPS levels. Antibiotic treatments increased the fecal LPS level in mice with tIsc more than vehicle treatment (**Figure 3F**).

tIsc operation itself caused mild colitis in control mice compared to those of control and sham mice (**Figures 3G,H**). Oral gavage of vancomycin or ampicillin significantly deteriorated tIsc-induced colitis; they dramatically increased colon shortening, myeloperoxidase activity, and TNF-α and IL-1β levels in the colon (**Figures 3G–J**). Their treatments also increased the CD11b$^+$/CD11c$^+$ and NF-κB$^+$/CD11c$^+$ cell (activated macrophage/dendritic cell) populations in the colon (**Figures 3K,L**).

Orally Delivered Fecal Transplantations of Vancomycin-Treated tIsc Mice Deteriorated tIsc-Induced Cognitive Impairment and Colitis in Mice

To understand whether the induction of cognitive impairment and colitis by antibiotic treatments was associated with gut dysbiosis, we orally transplanted the feces of sham mice into normal control mice (FC), tIsc-induced mice into tIsc mice (IFI), vancomycin-treated normal control mice into tIsc mice (IFV), or vancomycin-treated tIsc mice into mice with tIsc (IFIV) and examined their effects on the occurrence and development of cognitive impairment and colitis (**Figure 4A**). The occurrence of cognitive impairment by fecal transplantation of IFI into tIsc mice was not significantly different to that in mice with tIsc (**Figures 4B,C**). However, the fecal transplantation of IFV or IFIV into tIsc mice caused more severe cognitive impairment in Y-maze and novel object recognition tasks compared to those in control or IFI feces-transplanted mice. Orally delivered fecal transplantation of IFIV or IFV also significantly increased the NF-κB$^+$/Iba1$^+$ and LPS$^+$/Iba1$^+$ cell populations in the hippocampus (**Figures 4D,E**). Furthermore, treatment with IFIV or IFV increased the caspase 3$^+$/NeuN$^+$ (apoptotic neuron) cell population in the hippocampus, while the BDNF$^+$/NeuN$^+$ cell population was suppressed (**Figures 4F,G**). Orally delivered fecal transplantation of IFIV or IFV into mice also caused colon shortening and induced myeloperoxidase activity in the colon more severely than those of control and IFI feces-transplanted mice (**Figures 4H,I**). Treatment with IFIV or IFV also increased the CD11b$^+$/CD11c$^+$ and NF-κB$^+$/CD11c$^+$ cell populations in the colon (**Figures 4J,K**). Overall, the effect of IFV fecal transplantation on the cognitive impairment colitis was not significantly different with that of IFIV fecal transplantation.

Enterobacter xiangfangenesis Caused Cognitive Impairment in Mice With and Without tIsc

Oral administration of vancomycin significantly increased the *Enterobacter xiangfangenesis* population in mice with tIsc. Therefore, to understand whether *Enterobacter xiangfangenesis* was associated with the occurrence and development of cognitive impairment, we examined its effect on cognitive impairment in

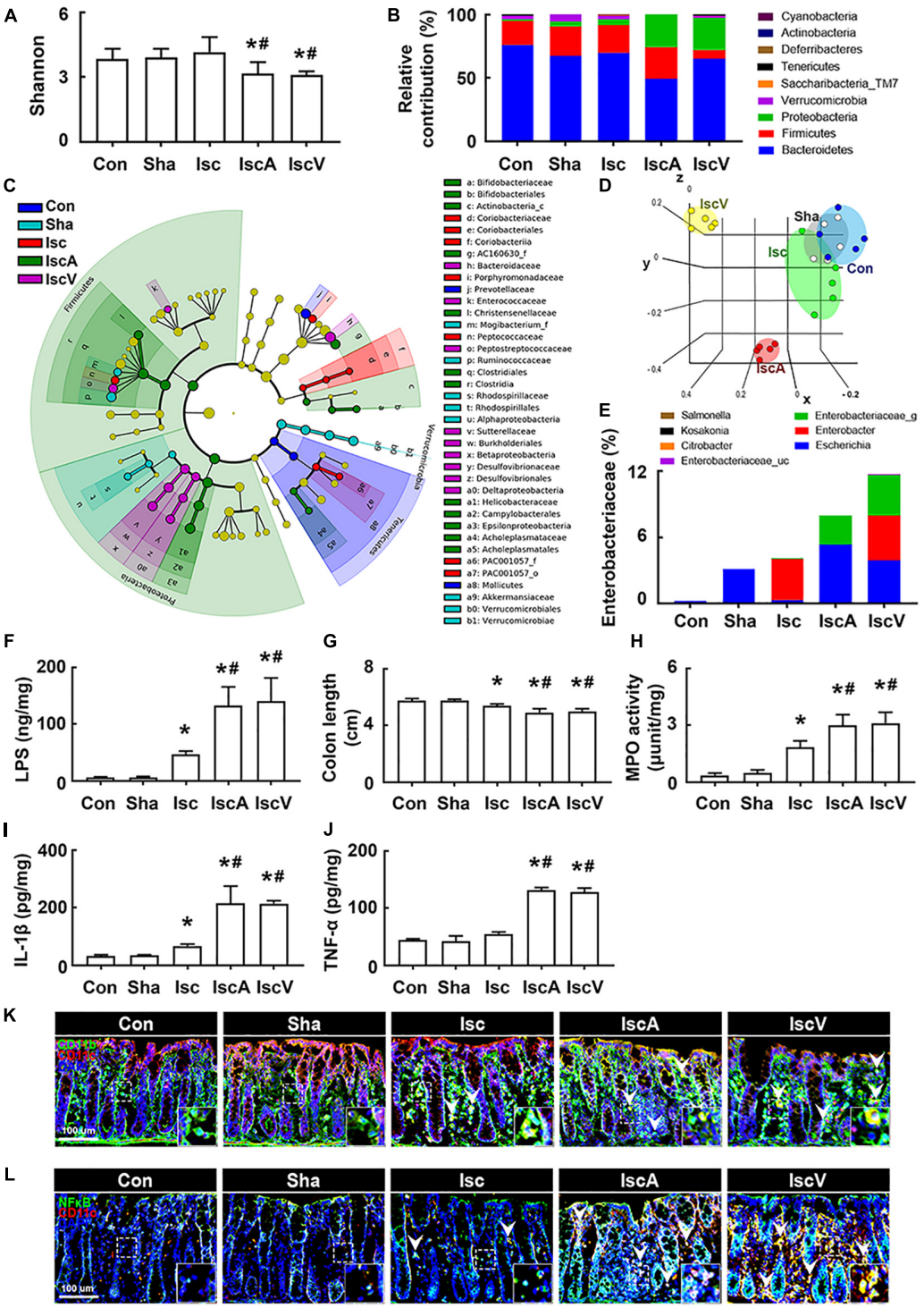

FIGURE 3 | Vancomycin and ampicillin caused the gut dysbiosis and colitis in mice with and without tIsc. Effects on the composition of gut microbiota, analyzed by the pyrosequencing: Shannon **(A)**, phylum **(B)**, cladogram **(C)** generated by LEfSE indicating significant differences in gut microbial abundances among Con (blue), Sha (gray), Isc (green), IscA (red), and IscV (yellow) groups, and principal coordinate analysis (PCoA) plots based on Jensen-Shannon analysis **(D)**. The threshold logarithmic score was set at 2.0 and ranked. Yellow nodes represent species with no significant difference. **(E)** Effects on bacterial population cultured in the DHL agar plate. **(F)** Effects on the fecal LPS level. Effects on colon length **(G)**, myeloperoxidase activity **(H)**, IL-1β **(I)**, and TNF-α expression **(J)** in the colon. Effects on $CD11b^{+}/CD11c^{+}$ **(K)** and $NF\text{-}\kappa B^{+}/Iba1^{+}$ cell **(L)** populations in the colon. Con, Sha, Isc, IscA, and IscV in the *x*-axis of figures indicate groups treated with vehicle in normal control mice, vehicle in sham mice, ampicillin in mice with tIsc, and vancomycin in mice with tIsc, respectively. All data were expressed as mean ± SD (**A–E**, $n = 5$; **F–J**, $n = 6$). $^{*}p < 0.05$ vs. Sha group. $^{\#}p < 0.05$ vs. Isc group.

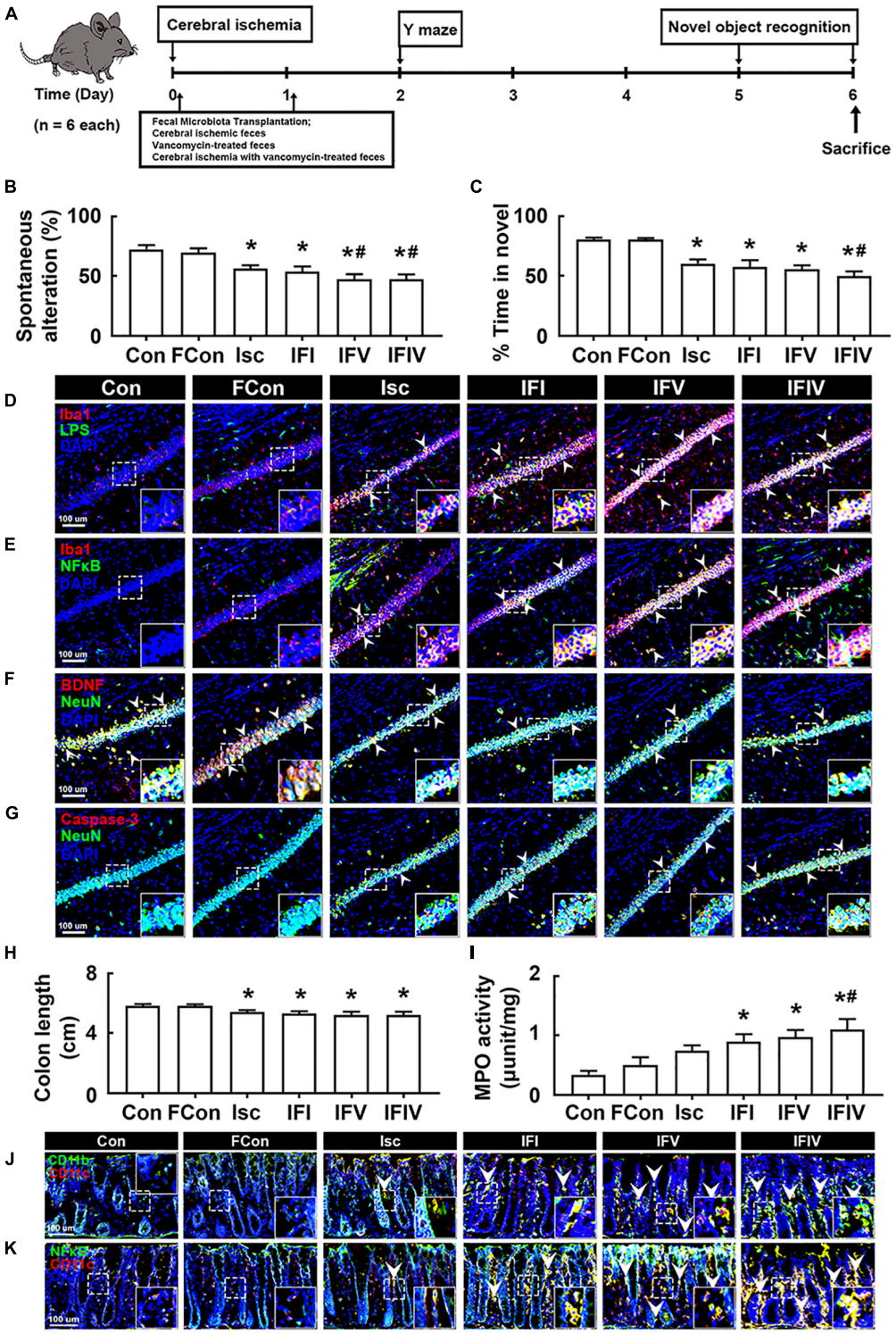

FIGURE 4 | The fecal transplantations of vancomycin-treated mice deteriorated the cognitive impairment and colitis in mice with tIsc. **(A)** Experimental protocol. Effects on the cognitive function, assessed by the Y-maze **(B)** and novel object recognition **(C)**. Effects on $LPS^{+}/Iba1^{+}$ **(D)**, $NF\text{-}\kappa B^{+}/Iba1^{+}$ **(E)**, $BDNF^{+}/NeuN^{+}$ **(F)**, and caspase $3^{+}/NeuN^{+}$ cell **(G)** populations in the hippocampus, assessed by a confocal microscope. Effects on colon length **(H)**, myeloperoxidase activity **(I)**, and $CD11b^{+}/CD11c^{+}$ **(J)** and $NF\text{-}\kappa B^{+}/Iba1^{+}$ **(K)** cell populations in the colon. Con, FC, Isc, IFI, IFV, and IFIV in the *x*-axis of figures indicate groups orally treated with vehicle into normal control mice, feces of sham mice into normal control mice, vehicle into mice with tIsc, Isc mouse feces into mice with tIsc, vancomycin-treated normal control mice into tIsc mice, and vancomycin-treated tIsc mouse feces into mice with tIsc, respectively. All data were expressed as mean ± SD ($n = 6$). $^{*}p < 0.05$ vs. FC group. $^{\#}p < 0.05$ vs. Isc group.

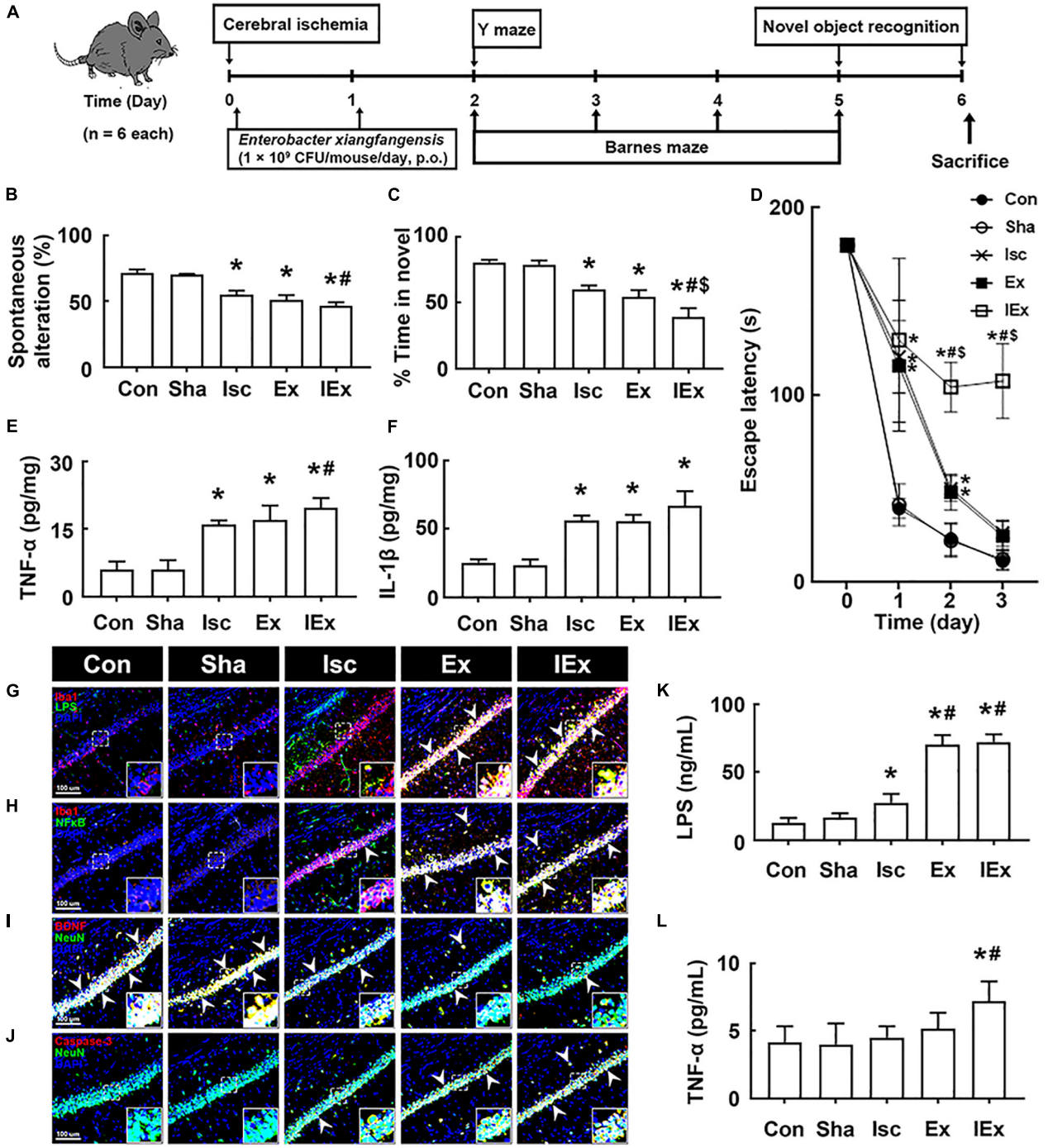

FIGURE 5 | *Enterobacter xiangfangenesis* caused the cognitive impairment in mice with and without tIsc. **(A)** Experimental protocol. Effects on the cognitive function, assessed by the Y-maze **(B)**, novel object recognition **(C)**, and Banes maze tasks **(D)**. Effects on TNF-α **(E)** and IL-1β expression **(F)** in the hippocampus, assessed by ELISA. Effects on $LPS^{+}/Iba1^{+}$ **(G)**, $NF\text{-}\kappa B^{+}/Iba1^{+}$ **(H)**, $BDNF^{+}/NeuN^{+}$ **(I)**, and caspase $3^{+}/NeuN^{+}$ cell **(J)** populations in the hippocampus, assessed by a confocal microscope. Effects on LPS **(K)** and TNF-α **(L)** levels in the blood. Con, Sha, Isc, Ex, and IEx in the *x*-axis of figures indicate groups treated with vehicle in normal control mice, vehicle in sham mice, vehicle in mice with tIsc, *Enterobacter xiangfangenesis* in control mice, and *Enterobacter xiangfangenesis* in mice with tIsc, respectively. All data were expressed as mean ± SD ($n = 6$). $^{*}p < 0.05$ vs. Sha group. $^{\#}p < 0.05$ vs. Isc group. $^{\$}p < 0.05$ vs. Ex group.

mice with and without tIsc in Y-maze, novel object recognition, and Banes maze tasks (**Figure 5A**). Oral gavage of *Enterobacter xiangfangenesis* severely deteriorated the cognitive impairment in mice with or without tIsc compared to those in control and sham mice (**Figures 5B–D**). *Enterobacter xiangfangenesis* treatment deteriorated cognitive impairment in mice with

tIsc more severely than in mice without tIsc. *Enterobacter xiangfangenesis* treatment also significantly increased TNF-α and IL-1β expression in the hippocampus (**Figures 5E,F**). Exposure of mice with tIsc to *Enterobacter xiangfangenesis* significantly decreased the $BDNF^+/NeuN^+$ neuron cell population in the hippocampus, while the caspase $3^+/NeuN^+$ (apoptotic neuron) cell and activated microglia ($LPS^+/Iba1^+$ and $NF\text{-}\kappa B^+/Iba1^+$ cells) populations increased (**Figures 5G–J**). Furthermore, treatment with *Enterobacter xiangfangenesis* increased LPS and TNF-α levels in the blood of mice with or without tIsc (**Figures 5K,L**).

Orally Gavaged Enterobacter xiangfangenesis Caused Colitis and Gut Microbiota Alteration in Mice With and Without tIsc

Next, we examined the effects of orally gavaged *Enterobacter xiangfangenesis* on gut microbiota composition and colitis in mice. The induction of tIsc in mice did not significantly shift the gut microbiota composition compared to that of *Enterobacter xiangfangenesis*-treated mice (**Figures 6A–D**, **Supplementary Figure S4B** and **Supplementary Tables S3**, **S4**). The exposure of mice with or without tIsc to *Enterobacter xiangfangenesis* fluctuated α-diversity and shifted β-diversity in the gut microbiota composition. Furthermore, treatment with *Enterobacter xiangfangenesis* increased the Firmicutes and Proteobacteria populations, while the Bacteroidetes population decreased. These treatments also increased the fecal LPS levels in mice with or without tIsc (**Figure 6E**).

Furthermore, exposure to *Enterobacter xiangfangenesis* induced colon shortening and myeloperoxidase activity in mice with and without tIsc (**Figures 6F,G**). The difference in *Enterobacter xiangfangenesis*-induced colon shortening and myeloperoxidase activity between mice with and without tIsc was not significant. Treatment with *Enterobacter xiangfangenesis* also upregulated TNF-α expression in mice with and without tIsc (**Figure 6H**). However, the IL-1β level was not affected (**Figure 6I**). Treatment with *Enterobacter xiangfangenesis* also increased the $NF\text{-}\kappa B^+/CD11c^+$ and $CD11b^+/CD11c^+$ cell populations in the colon (**Figures 6J,K**). *Enterobacter xiangfangenesis* treatment significantly deteriorated colitis in mice with tIsc.

DISCUSSION

Recently, a series of studies have highlighted that gut microbiota bidirectionally communicates with the brain and gut dysbiosis is closely associated with the occurrence of psychiatric disorders, including Alzheimer's disease (AD) (Bercik et al., 2011; Quigley, 2017; Rieder et al., 2017). The exposure of humans and animals to antibiotics, particularly oral administration, reduces bacterial diversity and induces antibiotic resistance, gut dysbiosis, and psychiatric disorders depending on the route of their administration: orally administered antibiotics such as ampicillin generally altered gut microbiota more severely than parentally injected ones (Zhang et al., 2013; Modi et al., 2014; Jang et al., 2018a; Zhou et al., 2020). Fröhlich et al. (2016) reported that oral gavage of an ampicillin, vancomycin, neomycin, bacitracin, and meropenem cocktail, which did not enter the brain, disrupted gut microbiota and impaired cognitive function in a novel object recognition task. Oral administrations of other antibiotic mixtures, such as a cocktail of ampicillin, vancomycin, metronidazole, neomycin, and amphotericin-B, also modified gut microbiota composition, caused anxiety, and/or impaired cognitive function in mice (Desbonnet et al., 2015; Hoban et al., 2016). Oral administration of ampicillin, which exhibits the same spectrum as amoxicillin *in vitro*, significantly caused colitis and anxiety in mice despite short-term treatment (Becattini et al., 2016; Jang et al., 2018a). However, oral administration of early life antibiotic penicillin decreased anxiety-like behavior in male mice (Leclercq et al., 2017). Oral administration of antibiotics (a cocktail of neomycin, bacitracin, and pimaricin) reduced anxiety in mice (Bercik and Collins, 2014). Nevertheless, to reduce the risk of pathogen infections, antibiotics are recommended in patients with ischemic brain injury (Xi et al., 2017). Therefore, to understand this discrepancy, the effects of the oral administration of vancomycin and ampicillin, which are recommended in patients with ischemic brain injury, on the occurrence of psychiatric disorders including cognitive impairment should be elucidated.

In the present study, we found that the oral administration of vancomycin or ampicillin, despite short-term treatment, markedly increased the fecal Proteobacteria population and LPS production in tIsc mice, and in normal control mice orally gavaged with ampicillin (Jang et al., 2018c). Jang et al. (2018c) also reported that oral administration of ampicillin significantly increased the Proteobacteria population and bacterial LPS production in mice. These data are consistent with previous reports that oral ampicillin (Zhang et al., 2013) and vancomycin (Isaac et al., 2017) severely disrupted the gut microbiota of healthy control mice. These results suggest that the oral administration of vancomycin and ampicillin can cause gut dysbiosis, whether in healthy or impaired conditions, and the application of these antibiotics by oral administration for the therapy of brain ischemia can severely increase gut microbiota LPS production with gut dysbiosis. We also found that these antibiotic treatments deteriorated colitis in mice with tIsc; they induced myeloperoxidase activity, active macrophages/dendritic cells ($CD11b^+/CD11c^+$ and $NF\text{-}\kappa B^+/CD11c^+$ cells) population, and suppressed gut tight junction protein expression in the colon. Furthermore, antibiotic treatment increased the blood LPS level and gut Proteobacteria population in tIsc mice more than in tIsc mice not treated with antibiotics. Jang et al. (2018a) reported that ampicillin treatment increased the blood LPS levels, gut Proteobacteria population, and absorption of orally gavaged fluorescein-conjugated dextran from the gut membrane into the blood in mice. These results suggest that oral administration of vancomycin and ampicillin can induce gut Proteobacteria population and bacterial LPS production and accelerate the absorption of fecal LPS into the blood by inducing gut dysbiosis-induced colitis (leaky gut) and suppressing gut tight junction protein expression. Furthermore, the induction of gut dysbiosis

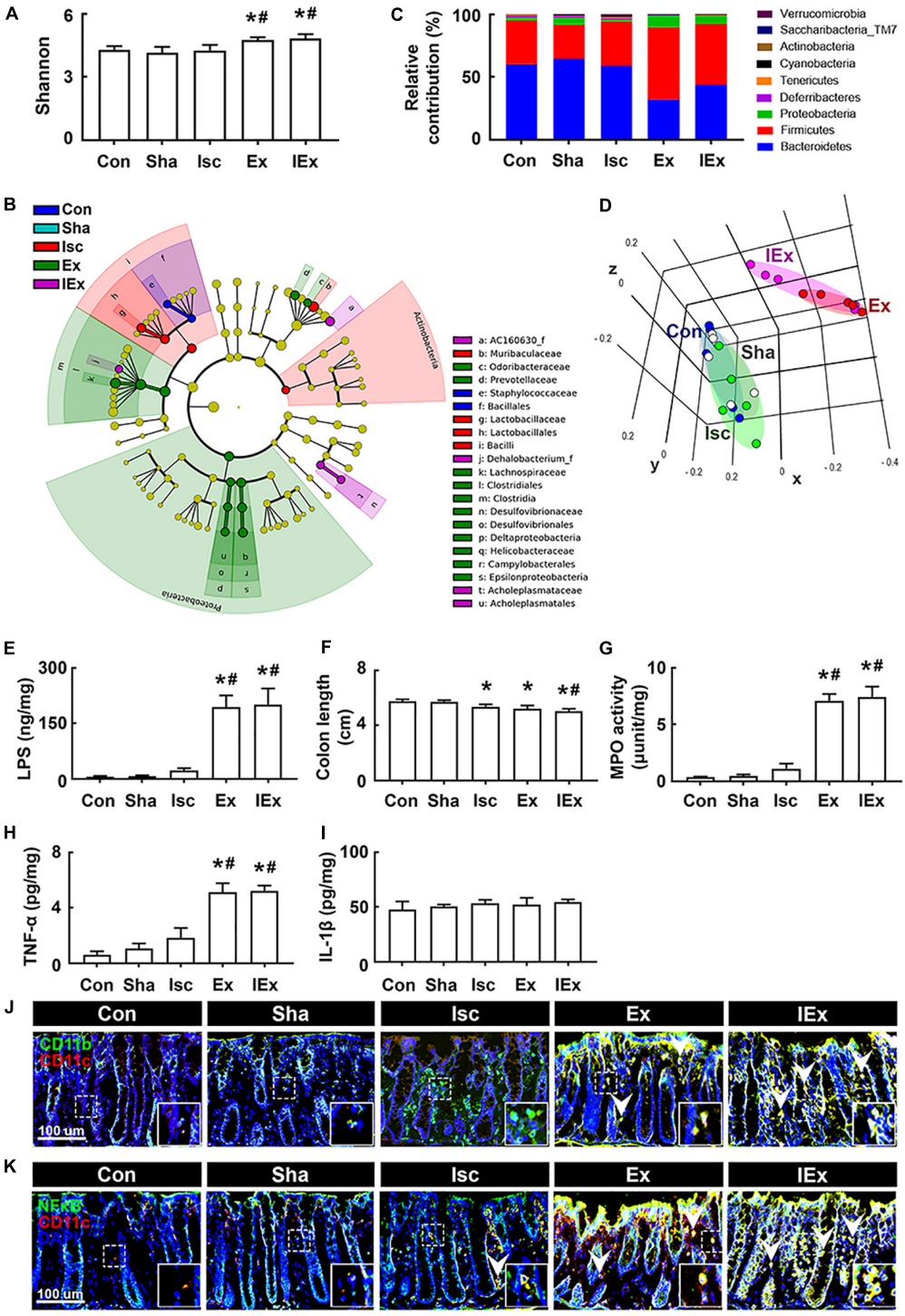

FIGURE 6 | *Enterobacter xiangfangenesis* caused the gut dysbiosis and colitis in mice with and without tIsc. Effects on the composition of gut microbiota, analyzed by the pyrosequencing: Shannon **(A)**, cladogram **(B)** generated by LEfSE indicating significant differences in gut microbial abundances among Con (blue), Sha (gray), Isc (green), Ex (red), and IEx (purple) groups, phylum **(C)**, and principal coordinate analysis (PCoA) plots based on Jensen-Shannon analysis **(D)**. The threshold logarithmic score was set at 2.0 and ranked in cladogram. Yellow nodes represent species with no significant difference. **(E)** Effects on the fecal LPS level. Effects on colon length **(F)**, myeloperoxidase activity **(G)**, and TNF-α **(H)** and IL-1β **(I)** expression in the colon. Effects on $CD11b^{+}/CD11c^{+}$ **(J)** and $NF\text{-}\kappa B^{+}/Iba1^{+}$ cell **(K)** populations in the colon. Con, Sha, Isc, Ex, and IEx in the *x*-axis of figures indicate groups treated with vehicle in normal control mice, vehicle in sham mice, vehicle in mice with tIsc, *Enterobacter xiangfangenesis* in control mice, and *Enterobacter xiangfangenesis* in mice with tIsc, respectively. All data were expressed as mean ± SD (**A–D**, $n = 5$; **E–I**, $n = 6$). $^{*}p < 0.05$ vs. Sha group. $^{\#}p < 0.05$ vs. Isc group.

by orally administered antibiotics, such as the induction of the Proteobacteria population by ampicillin or vancomycin, may elevate the translocation of LPS into the brain, particularly the hippocampus, resulting in neuroinflammation, as previously reported (Banks et al., 2015). Moreover, oral administration of antibiotics (vancomycin and ampicillin) increased activated microglial ($LPS^+/Iba1^+$ and $NF\text{-}\kappa B^+/Iba1^+$) cell populations, induced increased NF-κB activation, and suppressed the activated neuron ($BDNF^+/NeuN^+$) cell population in the hippocampus of tIsc mice. In addition, vancomycin treatment caused cognitive impairment in normal control mice. Jang et al. (2018a) reported that ampicillin treatment can cause psychiatric disorders and colitis in control mice. We also found that the number of neuron cells suppressed by antibiotics was inversely proportional to the number of $NF\text{-}\kappa B^+/Iba1^+$ cells in the hippocampus with tIsc and $NF\text{-}\kappa B^+/CD11c^+$ cells in the colon. The activated neuron ($BDNF^+/NeuN^+$) cell population was in inverse proportion to the activated microglial ($LPS^+/Iba1^+$ and $NF\text{-}\kappa B^+/Iba1^+$) cell populations in the hippocampus. The fecal LPS level was inversely proportional to tight junction protein expression. LPS recognition proteins such as toll-like receptor 4 are increased in the brains of patients with neuro-inflammation (Buchanan et al., 2010; Li et al., 2016). These results suggest that oral administration of vancomycin or ampicillin can increase the absorption of gut microbiota products including LPS through the overgrowth of the Proteobacteria population in the gut into the blood and brain, which can accelerate hippocampal inflammation, resulting in cognitive impairment.

While these data illustrated the profound impact of oral antibiotics on gut microbiota disruption and associated host responses, previous reports illustrated that changing ampicillin and vancomycin from oral to injective administration significantly alleviated the damages on gut microbiota (Zhang et al., 2013; Isaac et al., 2017; Zhou et al., 2020). These findings are consistent with the report by Vogt et al. (2016), showing that intraperitoneally injected minocycline does not display anxiolytic or anti-depressant behaviors in mice. Thus it is plausible that changing vancomycin and ampicillin administration from oral to injection may help mitigate the reported detrimental impact of the antibiotic treatment in tIsc hosts.

The fecal transplantations of vancomycin-treated mice severely deteriorated colitis in the transplanted tIsc mice compared to those in mice transplanted with mouse feces not treated with vancomycin; they induced colon shortening, myeloperoxidase activity, and infiltration of activated macrophages and dendritic cells ($CD11b^+/CD11c^+$ and $NF\text{-}\kappa B^+/CD11c^+$) into the colon. The transplantation of vancomycin-treated mouse feces, which contained a higher abundance of gut Proteobacteria than those of vancomycin-untreated mouse feces, also caused cognitive impairment and hippocampal inflammation compared to those in IFI feces-transplanted mice; it impaired the cognitive function and induced the hippocampal NF-κB-activated microglia cell population. However, the transplantation of control or tIsc mouse feces into tIsc mice did not deteriorate colitis and cognitive impairment. Furthermore, we found that treatment with the antibiotics vancomycin and ampicillin disrupted gut microbiota composition in mice with and without tIsc; they significantly increased the population of Proteobacteria including *Enterobacter xiangfangensis*. Treatment with vancomycin or ampicillin more severely caused gut dysbiosis in mice with tIsc than in mice without tIsc. Thus, they induced colon shortening and myeloperoxidase activity, activated macrophage and dendritic cell ($CD11b^+/CD11c^+$ and $NF\text{-}\kappa B^+/CD11c^+$) populations in the colon, impaired cognitive function, and reduced the $BDNF^+/NeuN^+$ cell population in the hippocampus and increased the activated microglia ($LPS^+/Iba1^+$ and $NF\text{-}\kappa B^+/Iba1^+$ cells) population. Atli et al. (2006) reported that orally administered amoxicillin caused adverse neurological side effects in children such as anxiety, hyperactivity, confusion, and behavioral changes. Jang et al. (2018a) also reported that oral administration of ampicillin or ampicillin-inducible *Klebsiella oxytoca* in the gut microbiota of mice caused gut microbiota disruption, including an increase in the Preteobacteria population, and hippocampal inflammation in mice. These results suggest that oral administration of the antibiotics vancomycin and ampicillin for the therapy of patients with brain ischemia can stimulate to facilitate the proliferation of the Proteobacteria population, such as *Enterobacter xiangfangensis*, which can cause gut and hippocampal inflammation and then accelerate the progression of cognitive decline. Antibiotics-inducible gut dysbiosis can affect the secretion of neuroactive, endocrinal, and immunological molecules in the intestine by inducing NF-κB activation by gut bacteria byproducts such as LPS, resulting in the deterioration of cognitive impairment progression.

In conclusion, treatment with the antibiotics vancomycin and ampicillin by oral administration deteriorated the cognitive impairment with gut dysbiosis in both healthy and tIsc mice (**Figure 7**). The finding has direct implications for clinic application of oral vancomycin and ampicillin in patients with

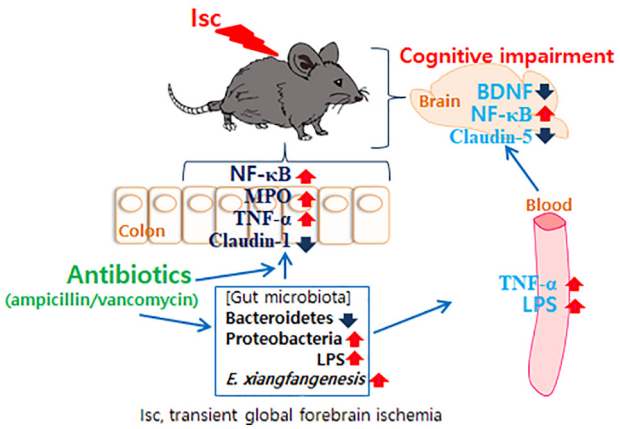

FIGURE 7 | Proposed mechanism of cognitive impairment with gut dysbiosis by antibiotics.

brain ischemia. While it is recognized that changing antibiotic administration from oral to injection alleviated gut microbiota disruption, its applicability in clinical treatment for patients with brain ischemia needs further validation.

MATERIALS AND METHODS

Culture of Gut Bacteria

For the culture of gut bacteria, the fresh feces of mice (approximately 0.1 g) was suspended in a ninefold volume of general anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan), diluted in a stepwise manner, and inoculated directly onto selective agar plates (Jang et al., 2018b). Deoxycholate and hydrogen sulfide lactose (DHL) agar (Nissui Pharmaceutical Co.) plates were aerobically cultured for 1 day at 37°C and blood liver (BL) agar (Nissui Pharmaceutical Co.) plates were anaerobically cultured for 3 days at 37°C.

Enterobacter xiangfangenesis was cultured in GAM broth. Briefly, isolated bacteria were anaerobically cultured in 0.3 L of GAM broth at 37°C (an optical density at 600 nm, 0.6–0.8), centrifuged at 5,000 *g* for 20 min, and washed with saline twice. Collected cells (5×10^9 colony forming unit [CFU]/mL) were suspended in 1% glucose (for *in vivo* study). Isolated bacteria were identified by Gram staining, a sugar utilization test, and 16S rRNA sequencing.

Animals

Male C57BL/6 mice (6 weeks old, 20–22 g) were obtained from the Orient Animal Breeding Center (Seoul, South Korea) and housed in controlled standard conditions. All mice were housed in plastic cages with a raised wire floor (3 mice per cage) at 20–22°C, 50 ± 10% humidity, and 12-h light/dark cycle (lights on from 07:30 to 19:30). They were fed standard laboratory chow and water *ad libitum*. Mice were used in the experiments after the acclimation for 1 week.

Preparation of Mice With tIsc and Treatment With Antibiotics

Mice with tIsc were prepared according to the method of Lee et al. (2016). Each group consisted of eight mice. Briefly, their bilateral common carotid arteries were occluded for 15 min. Mice were anesthetized with 2% isoflurane and maintained with 1% isoflurane maintenance during ischemia-induced surgery. In sham mice, the bilateral common carotid arteries were not occluded and only isolated from the adjacent vag us nerve. The tIsc- and sham-operated mice did not die during the experiment. Ampicillin (200 mg/kg/day) and vancomycin (150 mg/kg/day), which were converted from mouse doses to human equivalent doses (ampicillin, 1–2 g/60 kg/day; vancomycin, 0.5–2 g/60 kg/day) based on body surface area [animal dose (mg/kg) × 3/37] (Nair and Jacob, 2016), were orally gavaged daily for 2 days from 24 h after the occlusion.

To examine the effects of gut bacteria on the occurrence and development of the cognitive impairment in patients with brain ischemia, the suspension of mouse feces (2.5 mg/mouse/day) treated with or without vancomycin or *Enterobacter xiangfangenesis* (1×10^9 CFU/mouse/day) were orally gavaged once a day for 5 days in mice. Each group consisted of 6 mice. Animal experiments were conducted with two replicates. To exclude the effects of orally administered antibiotics in the feces of mice, fecal suspension was prepared as follows: fresh feces were collected on the 5th day after the final vancomycin treatment, suspended in GAM broth on ice, centrifuged at 2,000 *g* for 15 min at 4°C, washed with 1% glucose, and used as the fecal microbial suspension (25 mg/mL suspended in 1% glucose).

Memory-related behaviors were measured using a Y-maze, novel object recognition, and a Banes maze on the 5th day after treatment with fecal suspension or bacterial suspension. At the end of the behavioral test, all animals were sacrificed by CO_2 inhalation. Blood, brains, and colons were collected. The brain and colon were stored at −80°C for the assay of biochemical markers. For the immunohistochemistry assay, mice were transcardially perfused with 4% paraformaldehyde for brain and colon tissue fixation. Brains and colon tissues were post-fixed with 4% paraformaldehyde for 4 h, cytoprotected in 30% sucrose solution, frozen, and cut using a cryostat (Leica, Nussloch, Germany) (Kim et al., 2020).

Behavioral Tasks

The Y-maze task was carried out in a three-arm horizontal maze (40 cm long and 3 cm wide with 12-cm-high walls) (Jang et al., 2018b). A mouse was initially placed within one arm and the sequence of arm entries was recorded for 8 min. A spontaneous alternation was defined as sequential entries into three arms such as ABC, BCA, and CAB and was calculated as the ratio (%) of spontaneous to possible alternations. A novel object recognition task was performed in the apparatus consisting of a dark-open field box (45 × 45 × 50 cm), as previously reported (Lee et al., 2018). Briefly, in the first trial, a mouse was placed in the box containing two identical objects and the frequency of touching each object was recorded for 10 min. In the second trial conducted 24 h after the first trial, a mouse was placed in the box containing one of the old objects used in the first trial and a new object. Novel object recognition was calculated as the ratio of the number of times touching the new object to the sum of the touching frequencies. A Barnes maze task was performed in the apparatus consisting of a circular platform (89 cm in diameter) with 20 holes (5 cm in diameter) situated evenly around the perimeter and an escape box located under only one of the holes below the platform, as previously reported (Kim et al., 2020). Briefly, the training/acquisition phase finished after the mouse entered the escape box or after a maximum test duration (5 min), following which the mouse was allowed to stay in the escape box for 30 s and then moved to the cage. If the mouse failed to find the escape box within 5 min, it was led to the escape box. The task was performed for 5 consecutive days.

Immunofluorescence Assay

Immunostaining was performed, as previously reported (Jang et al., 2018b,c). Briefly, brain and colon tissue sections were washed with PBS, blocked with normal serum, incubated with

antibodies for Iba1 (1:200, Abcam), LPS (1:200, Millipore), NF-κB (1:100, Cell Signaling), CD11b (1:200, Abcam), CD11c (1:200, Abcam), and/or NeuN (1:200, Millipore) antibodies overnight, and treated with the secondary antibodies for 2 h. Secondary antibodies conjugated with Alexa Fluor 488 (1:200, Invitrogen) or Alexa Fluor 594 (1:200, Invitrogen) were then treated to enable visualization. Nuclei were stained with 4',6-diamidino-2-phenylindole, dilactate (DAPI, Sigma). Immunostained samples were scanned with a confocal laser microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

Brain and colon tissues were removed 2 h after the performance of the final task and homogenized with RIPA lysis buffer with a 1% protease inhibitor cocktail and a phosphatase inhibitor cocktail on ice (Jang et al., 2018a). The lysates were centrifuged at 10,000 *g* and 4°C for 10 min. For ELISA, the capture antibody for each cytokine was coated in a 96-well plate according to the manufacturer's protocol (eBioscience, San Diego, CA) and the resulting supernatants were transferred into the 96-wells. Thereafter the detection antibody solution was treated and measured the absorbance at 410 nm (Jang et al., 2018b).

Myeloperoxidase Activity and Limulus Amebocyte Lysate (LAL) Assays

Myeloperoxidase activity was measured, as previously reported (Jang et al., 2018a). Fecal and blood endotoxin contents were determined using the diazo-coupled LAL assay kit (Cape Cod Inc., E. Falmouth, MA), as previously reported (Kim et al., 2012).

Illumina iSeq Sequencing

Genomic DNA was extracted from the fresh stool of five mice (not transcardially perfused with 4% paraformaldehyde for brain and colon tissue sections) using a commercial DNA isolation kit (QIAamp DNA stool mini kit), as previously reported (Kim et al., 2020). The extracted genomic DNA was amplified using barcoded primers, which targeted the V4 region of the bacterial 16S rRNA gene. The sequencing for equimolar concentration of each amplicon was performed using Illumina iSeq 100 (San Diego, CA) (Kim et al., 2020). Reads taken from different samples were classified by unique barcodes of each polymerase chain reaction product and the target region in barcoded primers was identified. All of the linked sequences including adapter, barcode, and linker and low quality sequences (reads with two or more indefinite nucleotides, a low quality score, or < 500 bp) were eliminated. Potential chimeric sequences were confirmed by the Bellerophon formula. The taxonomic sorting of each read was assigned against the EzTaxon-e database[1], which has the 16S rRNA gene sequence of type strains that have valid published names and representative species level phylotypes of either cultured or uncultured entries in the GenBank database with complete hierarchical taxonomic classification from phyla to species. The 16S rRNA gene sequences originating from our study were deposited in NCBI's SRA (SRX4051778∼4051782, SRX3153173, SRX3153178∼3153180, SRX3153182). The species richness of samples was determined using the CLcommunity program. Subsampling was randomly performed to equalize the read size of tested samples to compare the different read size within tested samples. For the comparison of the estimated operational taxonomic units (OTUs) between tested samples, shared OTUs were obtained with the XOR analysis of the CLcommunity program. Pyrosequencing reads were deposited in the NCBI's short read archive under accession number PRJNA507690.

[1]http://eztaxon-e.ezbiocloud.net

Ethics Statement

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Kyung Hee University (IACUC Number: KUASP(SE)-18033) and performed according to the University Guide for Laboratory Animals Care and Usage.

Statistical Analysis

All experimental data are indicated as mean ± standard deviation (SD) (**Supplementary Table S1**) and conducted by GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA). The significance for data was analyzed using a non-parametric Mann Whitney test and one-way analysis of variance with *post hoc* Bonferroni's or Holm Sidak's multiple comparison test ($p < 0.05$). *p*-values in the present experiments are indicated in **Supplementary Table S2**.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Kyung Hee University.

AUTHOR CONTRIBUTIONS

K-EL and D-HK: conceptualization, writing – original draft, and writing – review and editing. K-EL and J-KK: experiment and data analysis. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.564271/full#supplementary-material

Supplementary Figure 1 | Average number of error hole visits during the Barnes maze test in **Figure 1D**.

Supplementary Figure 2 | Oral administration of vancomycin or ampicillin increased Iba1 + cell population in the hippocampus.

Supplementary Figure 3 | Oral gavage of vancomycin or ampicillin increased NF-κB$^+$/Iba1$^+$ **(A)**, LPS$^+$/Iba1$^+$ **(B)**, and caspase 3$^+$/NeuN$^+$ cell **(C)** populations and reduced BDNF$^+$/NeuN$^+$ cell population **(D)** in the hippocampus, assessed by a confocal microscope.

Supplementary Figure 4 | Antibiotics **(A)** and *Enterobacter xiangfangenesis* **(B)** caused gut alteration (LDA score) in mice.

Supplementary Table 1 | The gut microbiota composition ratio at the family level in ischemia-induced mice treated with or without antibiotics.

Supplementary Table 2 | The gut microbiota composition ratio at the genus level in ischemia-induced mice treated with or without antibiotics.

Supplementary Table 3 | The gut microbiota composition ratio at the family level in ischemia-induced mice treated with or without *Enterobacter xiangfangenesis*.

Supplementary Table 4 | The gut microbiota composition ratio at the genus level in ischemia-induced mice treated with or without *Enterobacter xiangfangenesis*.

Supplementary Table 5 | Means and SD of raw data in the experiments.

Supplementary Table 6 | *P*-values in the experiments.

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Exposure to One Antibiotic Leads to Acquisition of Resistance to Another Antibiotic *via* Quorum Sensing Mechanisms

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The vancomycin-resistant Enterococci (VRE) have progressively become a severe medical problem. Although clinics have started to reduce vancomycin prescription, vancomycin resistance has not been contained. We found that the transfer of vancomycin resistance in *Enterococcus faecalis* increased more than 30-fold upon treatment by streptomycin. Notably, treatment with an antibiotic caused the bacteria to become resistant to another. The response was even stronger in the well-studied plasmid pCF10 and the number of transconjugants increased about 100,000-fold. We tested four different antibiotics, and all of them induced conjugal response. Through a mathematical model based on gene regulation, we found a plausible explanation. *Via* quorum sensing, the change of the cell density triggers the conjugation. Moreover, we searched for generality and found a similar strategy in *Bacillus subtilis*. The outcome of the present study suggests that even common antibiotics must not be overused.

Keywords: dissemination of drug resistantance, selection pressure, nosocomial infection, conjugal genes, *prgX*, *prgQ*, post-antibiotic era, side effect

INTRODUCTION

The crisis of antibiotic resistance has received considerable attention in recent years (Chatterjee et al., 2011; Brown and Wright, 2016). Much effort has been made to uncover how antibiotic resistance is acquired (Baker et al., 2018). Such efforts have been made to avoid a post-antibiotic era where even common infections can once again lead to death. In the early-to-mid twentieth century, scientists already recognized that treatment with an antibiotic prompted bacteria to develop resistance to it. Moreover, administration of an antibiotic may induce efflux pumps (Papkou et al., 2020), which export antimicrobials and lead to multidrug resistance (Grimsey et al., 2020). To avoid accelerating antibiotic resistance, medical doctors are inclined to use early-discovered antibiotics. The other side effect of administrating antibiotics is that it kills not only the pathogens but also harmless bacteria. The collateral damage on the symbiotic bacteria may cause harm to a patient's health in the future (Blaser, 2016). Without the contribution of commensal bacteria to intestinal microbiota, patients are more vulnerable to infections (Buffie and Pamer, 2013). Regrettably, there is still another serious side effect of abusing antibiotics, and it has been long overlooked by the medical community. In the present study,

we found that administration of an antibiotic reduces the population of vulnerable cells in *Enterococcus faecalis*. Due to quorum sensing, the vulnerable donor cells spread another antibiotic resistance.

Enterococci are multiple drug-resistant nosocomial bacteria (Tendolkar et al., 2003). The opportunistic pathogen *E. faecalis* causes urinary tract infections and endocarditis (Paulsen et al., 2003). Typically, infections are present in patients who have an impaired immune system and who have received multiple courses of antibiotics (O'Driscoll and Crank, 2015). Shortly after the first isolation of vancomycin-resistant Enterococci (VRE) in 1988, they spread with unanticipated rapidity (Cetinkaya et al., 2000). Enterococci are proficient in the dissemination of genes that encode drug resistance (Arias and Murray, 2009). In *E. faecalis*, pheromone-inducible conjugation is a highly efficient process of transferring antibiotic resistance and virulence (Hirt et al., 2018). Plasmid pCF10 is one of the most well-studied conjugal systems (Hirt et al., 2005). The regulation of conjugal genes relies on the ratio of cCF10 to iCF10 (Chatterjee et al., 2013). The mating pheromone cCF10 is a heptapeptide (LVTLVFV). An inhibitor or a self-sensing signal iCF10 is another heptapeptide (AITLIFI). Both iCF10 and cCF10 are quorum-sensing signals. In **Figure 1**, the left shows that cCF10 and iCF10 are released by recipient and donor cells (Nakayama et al., 1994; Dunny, 2007), respectively. The donor cells uptake these two peptides through active transporters (Leonard et al., 1996). After importation, the peptides compete for PrgX. This protein is either bound with iCF10 to form $(PrgX\text{-}iCF10)_4$ or bound with cCF10 to form $(PrgX\text{-}cCF10)_4$ (Erickson et al., 2019). The ratio of donor to recipient cells determines the ratio of $(PrgX\text{-}iCF10)_4$ to $(PrgX\text{-}cCF10)_4$. The *prgX* and *prgQ* are the main regulatory genes of conjugal response (Shu et al., 2011). The *prgQ* and its downstream genes are in charge of conjugation, and the complex of PrgX protein regulates its expression. The $(PrgX\text{-}iCF10)_4$ represses the transcription initiation of *prgQ* by blocking the binding site of RNA polymerase but $(PrgX\text{-}cCF10)_4$ does not (Chen et al., 2017). In addition to the PrgX protein, the *prgX* gene utilizes sense-antisense interaction to repress the expression of *prgQ* because these two genes are in opposite directions with 223 nt overlapped (Chatterjee et al., 2011). The transcription of *prgX* produces truncated RNA named as Anti-Q, which terminates the transcription of *prgQ* and causes it to form Qs RNA (Shokeen et al., 2010). If the transcription of *prgQ* is not terminated by Anti-Q, it produces a longer RNA Q_L. Only when cells are induced by cCF10, the gene *prgQ* abundantly produces QL RNA (Bensing and Dunny, 1997). Both Qs and Q_L encode iCF10, but only Q_L triggers the expression of downstream conjugal genes (Chatterjee et al., 2011; Erickson et al., 2020). The hospital isolated vancomycin-resistant plasmid, pMG2200, also has *prgQ* and *prgX*, which regulates its conjugation. Namely, it utilizes the same regulatory strategy as pCF10 (Zheng et al., 2009). In the present study, we aim to discover how such conjugations respond to administration of an antibiotic.

MATERIALS AND METHODS

The Conjugation With the Presence of Antibiotics

For the conjugation of pCF10 shown in **Figure 2**, the recipients OG1SSp are resistant to 250 μg/ml spectinomycin and 1,000 μg/ml streptomycin (Hirt et al., 2005). The donors OG1RF (Torres et al., 1991) are resistant to 200 μg/ml rifampicin, and the plasmid pCF10 is resistant to 10 μg/ml tetracycline. Cells were cultured overnight in Todd-Hewitt Broth (THB) at 37°C. We then washed the cells twice and made a 1:10 dilution.

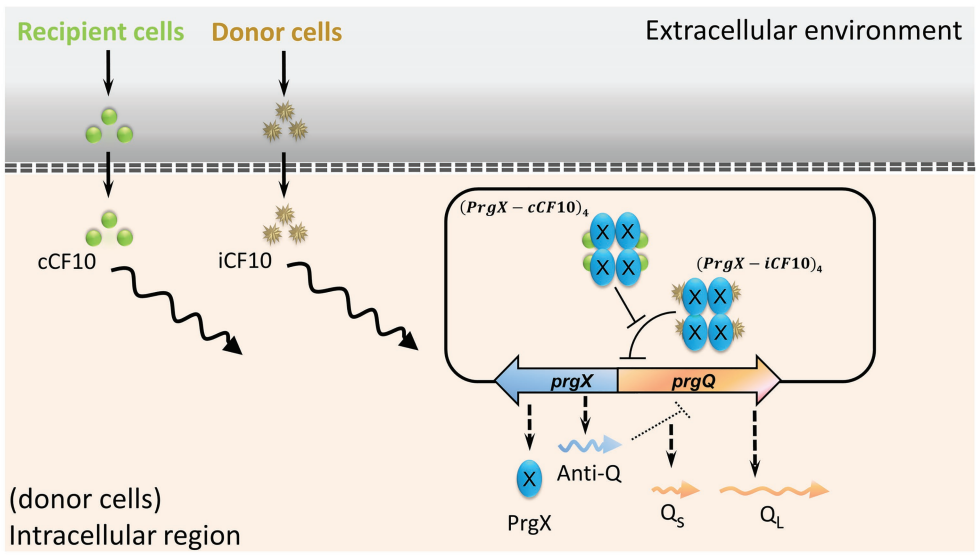

FIGURE 1 | The main regulation of conjugal genes in plasmid pCF10. Peptides cCF10 and iCF10, through PrgX protein, determine the expression level of *prgQ*. The induction of *prgQ* results in the production of Q_L RNA, which triggers the conjugation.

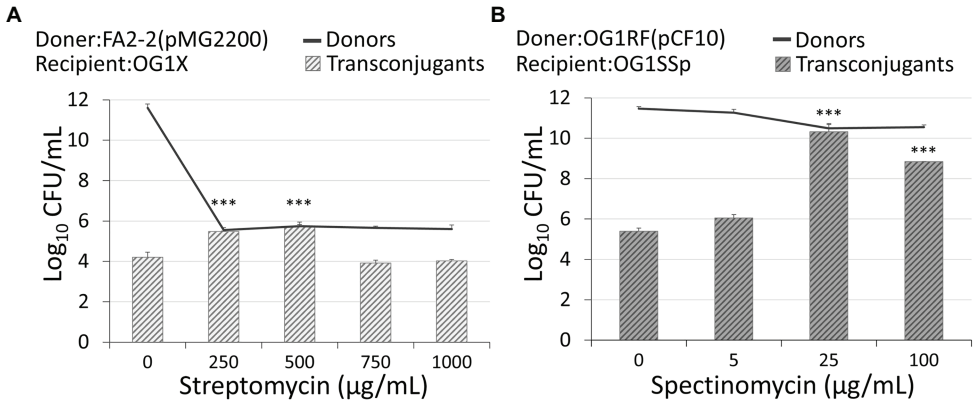

FIGURE 2 | Administration of antibiotics affects the conjugation rate. **(A)** An administration of streptomycin of 250 μg/ml or 500 μg/ml increased the conjugation of plasmid pMG2200 encoding vancomycin resistance. **(B)** An administration of spectinomycin of 25 μg/ml or 100 μg/ml leads to an increase in the conjugation of plasmid pCF10 (***indicates $p < 0.001$, in comparison to the case without an antibiotic).

Next, we treated separately donor cells and recipient cells with the spectinomycin concentration of 0, 250, 500, 750, or 1,000 μg/ml. After 60 min of incubation at 37°C, one volume of recipients was mixed with 10 volume of donors. The cells were continuously exposed to spectinomycin, and the mating lasted for 3 h. Serial dilutions of liquid samples were plated on agar. All data were collected from at least three bio-replicates; they are from different colonies while preparing overnight culture. While comparing two sets of data, we applied the permutation test (Fraker and Peacor, 2008) to obtain the *p*-value. The permutation test, from the original data sets, randomly generates replicate data and then calculates the *F*-value. The probability of any permutation data that produces an F-ratio more extreme than that of the actual data is the *p*-value.

For the conjugation of pMG2200 (Zheng et al., 2009) shown in **Figure 2**, the recipients OG1X (Ike et al., 1983; Flannagan et al., 2003) are resistant to 1,000 μg/ml streptomycin. The donors FA2-2 (Jacob and Hobbs, 1974; Flannagan et al., 2003) are resistant to 25 μg/ml rifampicin and the plasmid pMG2200 resistant to 10 μg/ml vancomycin. We cultured cells in THB at 37°C. We treated separately donors and recipients with the streptomycin concentration of 0, 5, 25, or 100 μg/ml. We then incubated cells overnight. We washed cells and took one volume of recipients mixed with five volume of donors. After 3 h of liquid mating, serial dilutions of samples were plated on agar. We prepared the plates of transconjugant cells with 1,000 μg/ml streptomycin and 10 μg/ml vancomycin. Note that we conducted negative control, and both pure donor and pure recipient cells were found with no colony on the plates. We also conducted such negative controls with proper antibiotics in other tests of conjugation to exclude the possibility of a spontaneous mutation.

The β-Galactosidase Assay of Plasmid pBK2 With the Treatment of Antibiotics

For the β-galactosidase assay shown in **Figure 3**, we use JRC101 instead of wild-type OG1RF. The difference between these two strains is that JRC101 (Chandler et al., 2005b) has a mutation of *ccfA* gene so it produces no cCF10. The reasons for using JCR101 are as follows. In nature, OG1RF(pCF10) produces little cCF10 because the PrgY protein encoded in the plasmid prevents cells from releasing cCF10 (Chandler et al., 2005a). However, plasmid pBK2 has no *prgY* gene (Shokeen et al., 2010). The gene map of pBK2 is in **Supplementary Figure S1**. Consequently, OG1RF(pBK2) produces the same amount of cCF10 as recipient cells. The extra cCF10 may severely change the conjugal response. To avoid it, we used JRC101 with plasmid pBK2. For the experiment with *Lactococcus lactis*, we transformed the pBK2 into strain MG1363 (Linares et al., 2010). *Enterococcus faecalis* and *L. lactis* were incubated overnight in THB at 37°C and in M17 at 32°C with the treatment of 5 and 30 μg/ml spectinomycin, respectively. Then, we induced cells with 0.5 μg/ml cCF10. After 90 min of incubation, we placed tubes on ice for 5 min and added 1/5 volume of toluene. The following β-galactosidase assays were performed as detailed by Miller (1972).

The steps for the assay of plasmid pBK2 with different cell densities (**Figure 4**) are as follows. After culturing JRC101(pBK2) overnight in THB at 37°C, we made a 1:10, 1:100, 1:1,000 or 1:10,000 dilutions. The normalized cell density is the cell density divided by the density of the overnight culture. After 2 h of incubation, we then induced cells with 30 ng/ml cCF10 for 90 min followed by β-galactosidase assay.

The Liquid Mating With Two Different Recipient Cells

For the liquid mating in **Figure 3C**, we use OG1RF (resistant to 200 μg/ml rifampicin) as donor cells harboring plasmid pCF10 (resistant to 10 μg/ml tetracycline). Two recipient cells are OG1Sp (Kristich et al., 2007; resistant to 1,000 μg/ml spectinomycin) and OG1X (resistant to 1,000 μg/ml streptomycin). After overnight culture in THB at 37°C, we washed cells twice and made a 1:10 dilution. We then separately treated donor cells and recipient cells with the streptomycin concentration of 0, 500, or 1,000 μg/ml.

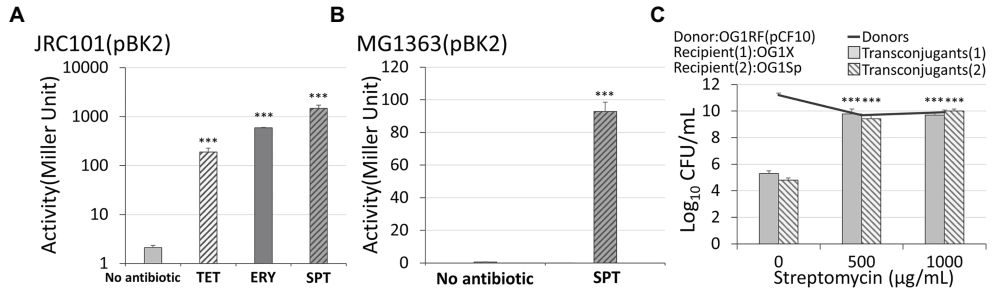

FIGURE 3 | The response of conjugal genes to different types of antibiotics. **(A)** In *Enterococcus faecalis* with plasmid pBK2, administration of 5 μg/ml spectinomycin, 5 μg/ml tetracycline, or 0.03 μg/ml erythromycin caused the conjugal response. **(B)** In *Lactococcus lactis* with plasmid pBK2, administration of spectinomycin results in the conjugal response. **(C)** An administration of streptomycin on the vulnerable donor cells OG1RF, resistant recipients OG1X, and vulnerable recipients OG1Sp increased the conjugation frequency. The transconjugant (1) and (2) are OG1X(pCF10) and OG1Sp(pCF10), respectively (***indicates $p < 0.001$, in comparison to the case without an antibiotic).

After 60 min of incubation at 37°C, we mixed one volume of each recipient with 10 volume of donors. We conducted liquid mating for 3 h. Serial dilutions of liquid samples were plated on agar.

The Mathematical Model

A mathematical model incorporating gene regulation of conjugal response in **Figure 1** is in the literature (Chatterjee et al., 2013). In the present study, we use exactly the same equations. We also apply exactly the same parameter values to the model, except for the volume conversion factor and the secretion rate constants. The volume conversion factor describes the volume of *E. faecalis* in the broth. We realized that the volume of *E. faecalis* is smaller than the volume that has been assigned in the original model. Due to the change of cellular volume, we modified the secretion rate constants. The **Supplementary Tables S1-S3** listed equations, nomenclatures, and parameter values. Briefly, the equations start with a variable describing the DNA configuration of *prgQ-prgX* region in donor cells. If DNA is bound with $(PrgX\text{-}iCF10)_4$, the transcription rate constant of *prgQ* is low. Oppositely, if cells are dominated by $(PrgX\text{-}cCF10)_4$, *prgQ* is highly expressed. The configuration of DNA also affects the transcription rate constant of *prgX*, which produces PrgX protein and truncated RNA in *prgX* direction, Anti-Q. Note that Anti-Q and the mRNA of PrgX are in different regions. The mRNA of PrgX is not under the effect of the truncated RNA in *prgQ* direction but Anti-Q is. To determine the concentration of Anti-Q, the model has to calculate the amount of the truncated RNA in *prgQ* direction and account for its influence on Anti-Q. In model, Anti-Q interferes in the transcription of *prgQ* to separate Qs from Q_L. Both Qs and Q_L produce iCF10. Note that recipient cells produce cCF10. The iCF10 and cCF10 in donor cells compete for PrgX protein to form $(PrgX\text{-}iCF10)_4$ and $(PrgX\text{-}cCF10)_4$, respectively. Thereby, the concentration of iCF10 and cCF10 controls the configuration of the DNA. The generation of conjugation protein is then calculated from the concentration of Q_L. In the present study, we use Q_L to indicate the conjugal level.

The Liquid Mating With Different Ratio of Donor to Recipient Cells

For conjugation of plasmid pCF10, we used OG1RF (Torres et al., 1991) and OG1SSp (Torres et al., 1991) as donor and recipient cells, respectively. After culturing overnight in THB at 37°C, we washed cells twice and made a 1:10 and a 1:100 dilution for high and low donor density, respectively. We treated recipient cells with the same procedure as that of high donor density. After 1-h incubation, we mixed one volume of recipients with 10 volume of donors and allowed the mating to last for 3 h. Serial dilutions of liquid samples were plated on agar. For the conjugation of plasmid pMG2200, we used FA2-2 and OG1X as donor and recipient cells, respectively. It followed the same procedure except that we made a $1{:}10^7$ dilution for low donor density.

The Information of Strains and Plasmids

From the lab of Prof. Gary M. Dunny in the University of Minnesota, we obtained *E. faecalis* strains OG1RF, JRC101, OG1SSp, OG1Sp, and OG1X and plasmids pCF10 and pBK2. The *E. faecalis* strain FA2-2 and the plasmid pMG2200 are from the lab of Prof. Haruyoshi Tomita in the Gunma University. The *L. lactis* strain MG1363 is from the lab of Prof. Cheng-Kang Lee in National Taiwan University of Science and Technology.

RESULTS

Dissemination of Vancomycin Resistance

In rifampicin-resistant donor cells, FA2-2, conjugal plasmid pMG2200 (Zheng et al., 2009) encodes vancomycin resistance. Through conjugation in liquid mating, streptomycin-resistant recipients OG1X acquired pMG2200 and became vancomycin-resistant transconjugants. The methods and strain information are detailed in section Materials and Methods. Without administration of streptomycin in donor cells, 1.6×10^4 colony forming units (CFUs) per milliliter of transconjugants were identified (first left bar in **Figure 2A**). While maintaining the environmental concentration of streptomycin at 250 μg/ml, the conjugation increased significantly. Specifically, when the

concentration of streptomycin reached 500 μg/ml, the number of transconjugants increased to 5.28×10^5 CFU/ml (**Figure 2A**). Compared with the case that did not receive treatment with streptomycin, the number of transconjugants increased more than 30-fold. The donor density decreased from 4.02×10^{11} to 4.63×10^5 CFU/ml (**Figure 2A**, black line). In other words, the conjugation rate per donor increased by more than 10 million times. This result is not from the spontaneous mutation of donor cells becoming streptomycin resistance because we treated donor cells with streptomycin without recipients, and we found no colony on the plate of transconjugants. **Figure 2A** suggests that the administration of streptomycin promoted the conjugal response of vancomycin-resistant plasmids, which increased the number of VRE. When further increasing the concentration of streptomycin, it is highly likely that vulnerable donors lose vitality, causing the number of transconjugants to decrease.

Conjugal Response of pCF10

The plasmid pMG2200 contains part of the pheromone-responsive genes *prgX* and *prgQ* identical to those in the plasmid pCF10 (Hirt et al., 2005; Zheng et al., 2009). If these genes are responsible for the induction of conjugation during treatment with antibiotics, the plasmid pCF10 should behave similarly to pMG2200. We allowed rifampicin-resistant donors, OG1RF, to transfer plasmid pCF10 (resistant to tetracycline) to the recipients (spectinomycin- and streptomycin-resistant OG1SSp). **Figure 2B** shows that the treatment with spectinomycin over 4 h considerably increased the conjugation frequency. Interestingly, not only the streptomycin but also spectinomycin stimulated the transfer of plasmids. When the concentration of spectinomycin was raised from 0 to 25 μg/ml, the number of transconjugants increased significantly from 2.52×10^5 to 2.11×10^{10} CFU/ml. The number of transconjugants increased about 100,000-fold. When we further increased the concentration of spectinomycin to 100 μg/ml, the number of transconjugants decreased to 7×10^8 CFU/ml. The trend is similar to that for pMG2200. Next, we examined the influence of a longer treatment with antibiotics. Compared with the case receiving no treatment, cells treated with antibiotics achieved a much higher conjugation frequency (2.85×10^9 CFU/ml of transconjugants) after exposure to 25 μg/ml spectinomycin for up to 20 h (**Supplementary Text S1** and **Supplementary Figure S2**).

Main Regulatory Genes *prgX* and *prgQ*

To confirm that genes *prgX* and *prgQ* are responsible for the conjugal behaviors in **Figure 2**, we used an engineered plasmid, pBK2. In pBK2 (**Supplementary Figure S1**), the only genes from the plasmid pCF10 are *prgX* and *prgQ*. The backbone of pBK2 is pCI3340 (Hayes et al., 1990), a plasmid in *L. lactis*. In pCF10, Q_L RNA leads to the expression of downstream conjugal genes, and thus it serves as an indicator of conjugation (Chatterjee et al., 2013). In pBK2, a reporter *lacZ* gene replaced the part of conjugal gene *prgQ* that encodes Q_L RNA (Breuer et al., 2017). Thereafter, we measured the beta-galactosidase activity to quantify the level of conjugation (Shokeen et al., 2010). In this simplified system, cells harboring pBK2 are imitators of donor cells. As for recipient cells, they secreted pheromone cCF10, so we added synthetic peptide cCF10 to mimic them.

When exposed to 0.5 μg/ml of the pheromone cCF10, plasmid pBK2 was uninduced if no antibiotics were administered (the left first column of **Figure 3A**). When the cells were treated with 5 μg/ml of spectinomycin from overnight culture, the beta-galactosidase activity increased significantly (the right first column of **Figure 3A**). This result is consistent with the conjugal behaviors of the plasmid pCF10 (**Figure 2B**). Note that the expression level may be a little sensitive to the experimental conditions, but the treatment with antibiotics led to a great induction of cells (**Supplementary Text S2** and **Supplementary Figure S3**). To further exclude the influence from other genes in *E. faecalis*, we transformed plasmid pBK2 into *L. lactis*, MG1363. Remarkably, the treatment with 30 μg/ml of spectinomycin significantly increased the expression level of *prgQ*, even in *L. lactis* (**Figure 3B**). This result suggests that genes *prgX* and *prgQ* are responsible for the induction of the conjugal response by antibiotics.

Treatment With Different Antibiotics

The antibiotics shown in **Figures 2A,B** are streptomycin and spectinomycin, respectively. The results imply that the induction of the conjugal response is not restricted to only one type of antibiotic. To confirm this, we treated cells harboring pBK2 with 5 μg/ml of tetracycline, and cells were induced (**Figure 3A**). Similarly, we treated cells with 5 μg/ml of erythromycin, but it is too much. We then reduced the concentration of erythromycin to 0.03 μg/ml and cells were induced (**Figure 3A**). The result suggests that the conjugal genes in donor cells can be induced by various antibiotics. To understand whether the vulnerable recipient cells might compromise the conjugation, we used OG1RF (pCF10) as donor cells and two recipient cells are OG1X and OG1Sp, which are resistant to streptomycin and spectinomycin, respectively. We then administered different concentrations of streptomycin (**Figure 3C**). Remarkably, streptomycin induced the conjugal response in donor cells without hindering the vulnerable recipient cells from acquiring plasmids. The conjugation increased more than 10,000-fold even for the recipient OG1Sp, which is sensitive to streptomycin. This outcome suggests that the type of antibiotic administered may not be important. Treatment with an antibiotic helps *E. faecalis* to become resistance to another antibiotic.

Insights From the Mathematical Model

We applied a mathematical model to describe the conjugal regulation of genes *prgX* and *prgQ* and used the amount of Q_L to quantify the expression level of the conjugal gene (Chatterjee et al., 2013). The results are shown in **Figure 4A**. In the y-axis, we use normalized Q_L, which are the expression levels divided by the lowest one. We obtained the lowest value of Q_L at a cell density of 10^{11} CFU/ml. Note that we use the inverted scale of the x-axis in **Figure 4**. The simulation results indicate that the expression level increased when donor density decreased. To confirm it, we examined the influence of cell density on the beta-galactosidase activity in pBK2 (**Figure 4B**). The normalized cell density is the cell density divided by the overnight cell density. The outcome of the mathematical model (**Figure 4A**) is consistent with experimental observations (**Figure 4B**). From the results of the mathematical model and

the expression level of the conjugal response in pBK2, we realized that low donor density stimulates the expression of the conjugal gene, thus facilitating the dissemination of plasmids. This is because donor cells release a quorum-sensing signal, iCF10, to suppress the conjugal response. Treatment with an antibiotic lowered cell density and thereby prompted the transfer of the plasmid that encodes resistance to another antibiotic.

Influence of Donor Density

To further confirm that donor density is the cause of antibiotic-induced conjugal response, we examined the influence of donor density on conjugation. We conducted liquid mating by mixing the same amount of recipient cells with either a high or low density of donor cells. We did different dilutions to overnight culture. For FA2-2(pMG2200), the dilutions of donor cells were 1:10 and 1:10,000,000. For OG1RF(pCF10), the dilutions of overnight culture were 1:10 and 1:100. We chose these values based on the donor cell density illustrated in **Figure 2**. In **Figure 5**, the gray and black columns display the number of transconjugant cells for the plasmids pMG2200 and pCF10, respectively. As expected, systems with low donor density appeared to have a high conjugation frequency. This outcome is consistent with **Figure 2**, where transconjugant cells increase with a decrease in donor density (lines in **Figure 2**), as long as the concentration of the antibiotic is not too high.

DISCUSSION

In addition to pMG2200, many other plasmids have the same regulatory genes. From National Center for Biotechnology Information (NCBI) blast results, plasmids pE512, EF62pB, pN48037F-2, and Efsorialis-p2, have identical sequences (**Supplementary Table S4**) as *prgX* and *prgQ* in pCF10. The conclusion of this study might also apply to these plasmids. Chatterjee et al. (2013) revealed that the plasmid pCF10 is capable of sensing its own density through the quorum sensing signal iCF10 encoded in *prgQ* and thus the conjugation frequency decreases at a high donor density. In the present study, we discovered that the administration of antibiotics lowers donor density but failed to stop the plasmid spread. Thus, cells treated with an antibiotic were induced to spread resistance to other types of antibiotics. Other plasmids in *E. faecalis* may also have a similar regulatory pattern as that of pCF10. Both plasmids pAD1 and pAM373 showed a decrease of conjugation frequency at a high donor density (Bandyopadhyay et al., 2016). Plasmids pMG2201 (Zheng et al., 2009) and pTEF1 (Paulsen et al., 2003) have been reported to have the same self-sensing signal and the same conjugal response as pAD1. All these plasmids might have the same response to administration of antibiotics. Recently, the dissemination of linezolid resistance through the plasmids with

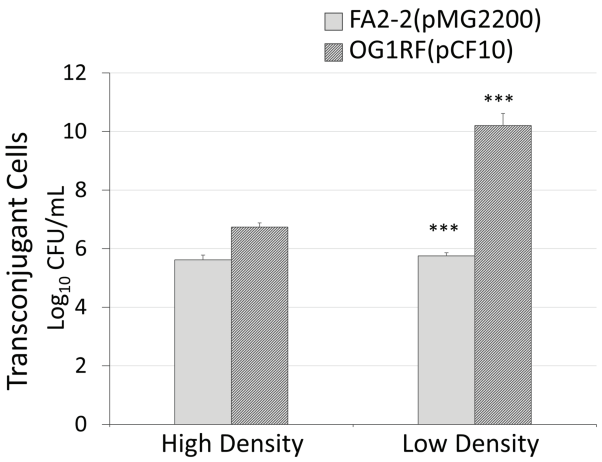

FIGURE 5 | Low donor density leads to a high conjugation rate. In x-axis, high density is a 1:10 dilution to the overnight donor culture and low density are 1:10,000,000 and 1:100 to an overnight donor culture of FA2-2 (pMG2200) and OG1RF (pCF10), respectively (***indicates $p < 0.001$, in comparison to the case with high cell density).

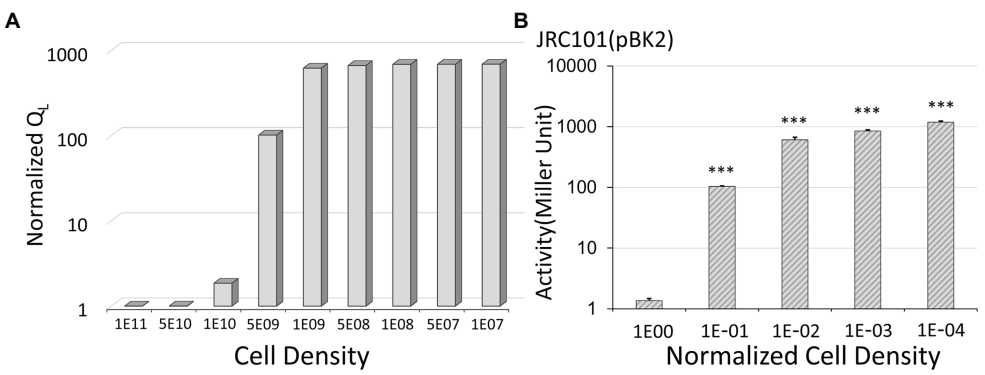

FIGURE 4 | The effect of cell density on conjugal response. **(A)** The simulation results show that low donor density leads to a high expression level. Note that we use the inverted scale of the x-axis. For the y-axis, the normalized Q_L is the expression levels divided by the lowest one obtained at a cell density of 10^{11} CFU/ml. **(B)** In *Enterococcus faecalis* with plasmid pBK2, low cell density results in the high expression of the conjugal gene. For the x-axis, the normalized cell density is the cell density divided by the overnight cell density (***indicates $p < 0.001$, in comparison to the case with a normalized cell density of one).

conjugal systems similar to that of pCF10 and pAD1 has also been found (Zou et al., 2020). Enterococci is notorious for nosocomial infection (Bereket et al., 2012), and the present study indicated that administration of a common antibiotic led to the incremental dissemination of plasmids encoding resistance to other antibiotics, including vancomycin. Thereby, it is better to stop overusing antibiotics, even the common ones! Note that not only the donor cells of *E. faecalis* are capable of sensing their own density through a quorum-sensing signal, but also the donors of *Bacillus subtilis* (Itaya et al., 2006) release a quorum-sensing signal phr^{*}_{LS20} to inactive the conjugation (Singh et al., 2013). Such a quorum-sensing strategy is not unique in *E. faecalis*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

C-CS initiated the idea, designed the experiment, and formulated the model. W-CC, Y-DC, and J-NC conducted the experiment. Y-SH repeated the experiment. F-YL and C-XY performed the simulation and analyzed the data. C-CS and EHW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articless/10.3389/fmicb.2020.580466/full#supplementary-material

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MINI REVIEW
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Evolution of Bacterial Tolerance Under Antibiotic Treatment and Its Implications on the Development of Resistance

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Recent laboratory evolution studies have shown that upon repetitive antibiotic treatments, bacterial populations will adapt and eventually became tolerant and resistant to the drug. Drug tolerance rapidly evolves upon frequent, intermittent antibiotic treatments, and such emerging drug tolerance seems to be specific to the treatment conditions, complicating clinical practice. Moreover, it has been shown that tolerance often promotes the development of resistance, which further reinforces the need of clinical diagnostics for antibiotic tolerance to reduce the occurrence of acquired resistance. Here, we discuss the laboratory evolution studies that were performed to track the development of tolerance in bacterial populations, and highlight the urgency of developing a comprehensive knowledge base of various tolerance phenotypes and their detection in clinics. Finally, we propose future directions for basic research in this growing field.

Keywords: tolerance, resistance, persistence, laboratory evolution, antibiotic

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DISTINCTION BETWEEN BACTERIAL TOLERANCE AND RESISTANCE AS SURVIVAL MECHANISMS TOWARD ANTIBIOTIC TREATMENT

Bacterial cells have a multitude of ways to fight against antibiotic assault. The most well-studied one is resistance (D'Costa et al., 2006), in which the bacteria possess genetic mutations that protect themselves against certain types of antibiotics, thus allowing them to grow at higher antibiotic concentrations. Mechanisms for resistance include direct inactivation of the drug, alterations of drug targets to reduce binding affinity, decreasing uptake or increasing efflux, redundant pathways to bypass the affected drug targets, and many more (Giedraitienė et al., 2011; Cox and Wright, 2013). In general, resistance directly counters the antibiotic's action mechanism. The most common detection method for resistance is through the measurement of the minimum inhibitory concentration (MIC), which is the lowest concentration that would kill or inhibit the growth of bacteria (Wiegand et al., 2008). A resistant population would have an elevated MIC. If the resistance phenotype only occurs in a subpopulation of cells, then it is known as heteroresistance. Heteroresistance is often unstable (Andersson et al., 2019): when grown in the absence of antibiotics within a limited number of generations, the phenotype reverts to susceptibility, while in the presence of antibiotic, the resistant subpopulation would rapidly outcompete the sensitive cells,

causing an ambiguous classification of the population as resistant. Unstable heteroresistance can be caused by an intrinsic instability of the resistance mutation itself, or by genetically stable mutations that confer high fitness cost. In the former case, the resistant subpopulation contains an increased copy number or tandem amplification of genes that increase resistance. In the latter case, resistance mutations with high fitness cost often drive the selection of second-site compensatory mutations when grown in the absence of antibiotic pressure, which will reduce the cost, but also lead to the loss of resistance. Although the majority of the population will become susceptible (harboring both resistance mutation and compensatory mutation which increase fitness), there will still be a small fraction of resistant cells (with reduced fitness, containing only the resistance mutation).

Another way for bacteria to survive antibiotic assault is tolerance. A tolerant population exhibits no difference in the MIC compared to a susceptible population, but can survive high doses of bactericidal antibiotics, often much higher than the MIC (Brauner et al., 2016; Balaban et al., 2019). Unlike resistance that allows the cells to grow under higher antibiotic concentrations, tolerant populations cannot grow nor replicate during treatment, and are just being killed at a lower rate. Tolerance can be quantified by measuring the minimum duration of killing (MDK) of the population, namely the time it takes to reduce the population by a certain percentage (e.g., 99%) at a certain dose of antibiotic (Fridman et al., 2014). If the tolerance phenotype only occurs in a subpopulation of cells, then it is known as persistence or heterotolerance (Lewis, 2010; Brauner et al., 2016). The tolerant subpopulation, called persisters, are present naturally in almost every bacterial populations. Persistence is known to be a phenotypic state rather than a genetic trait (Sulaiman et al., 2018), and is often interpreted as a bet-hedging strategy of bacteria to position some "seed" cells in a population to survive and outlive unfavorable environmental conditions (Lennon and Jones, 2011). Several mechanisms and pathways have been implicated in the phenotypic switch to a persister state, such as the stringent response and (p)ppGpp signaling, RpoS and the general stress response, SOS response, bacterial communication and quorum sensing, and Toxin/Antitoxin (TA) modules (Harms et al., 2016; Michiels et al., 2016b). More recently, it was proposed that the mechanistic basis of persister formation and resuscitation is via ppGpp ribosome dimerization (Song and Wood, 2020; Wood and Song, 2020). In short, ppGpp directly generate persister cells by inactivating ribosomes through stimulation of the ribosome modulation factor (Rmf), hibernation promoting factor (Hpf), and ribosome-associated inhibitor (RaiA). Upon addition of nutrients and removal of stress, cAMP levels are reduced and HflX is produced, causing the dissociation of inactive ribosomes into active ribosomes which leads to the resumption of growth. However, the exact mechanism of how persisters survive high-dose antibiotic treatment remains an open question and is likely antibiotic-dependent, though it is surmised that it can be partially ascribed to dormancy, which renders the killing mechanisms of many antibiotics ineffective (Wood et al., 2013; Kotte et al., 2014). Although persister cells do not differ genetically from their susceptible counterparts in the same population, it has been shown that the "level of persistence" – namely, the propensity of a strain to form persisters – can be modulated by genetic changes (Moyed and Bertrand, 1983; Germain et al., 2013). The presence of persisters explains the biphasic killing pattern when bacteria are treated with bactericidal antibiotics. The first phase with the steeper slope marks the rapid decline of the susceptible cells, and the second phase indicates the slow decline of the persister cells (Sulaiman and Lam, 2019).

LABORATORY EVOLUTION TO STUDY THE DEVELOPMENT OF TOLERANCE IN BACTERIA

Bacteria are well-known for their ability to adapt to different environmental conditions. When subjected to transient stresses, subpopulations of cells with favorable phenotypic niches that are otherwise outcompeted under normal conditions (such as those that have a slower growth), may thrive. This subpopulation may possess genetic mutations that confer tolerance or a higher level of persister formation, but with a certain fitness cost associated with the higher fraction of persister cells. When similar stresses are applied repeatedly, these cells could be selected, leading to an increase in the level of tolerance of the population over time. Therefore, the resulting evolved population may have different physiology and behavior compared to the original one. Recently, there have been substantial efforts devoted to study this adaptation mechanism and the development of tolerance in bacteria through laboratory evolution experiments, where bacterial populations are repetitively treated with high doses of antibiotics, mimicking clinical conditions (Fridman et al., 2014; Mechler et al., 2015; Michiels et al., 2016a; Van den Bergh et al., 2016; Khare and Tavazoie, 2020; Sulaiman and Lam, 2020a). One of the first experiments that inspired the laboratory evolution strategy came from Moyed et al. They repetitively treated *E. coli* cells with ampicillin and identified the *hipA* gene that confers a high persistence phenotype (Moyed and Bertrand, 1983). The increased tolerance upon laboratory evolution experiment was not only observed in *E. coli,* but also in *Staphylococcus aureus* (Mechler et al., 2015) and other ESKAPE pathogens (Michiels et al., 2016a), indicating the seemingly universal adaptability of bacteria toward intermittent antibiotic treatments. These laboratory evolution experiments, combined with theoretical models (Kussell et al., 2005; Patra and Klumpp, 2013), suggested that such cyclic antibiotic treatment protocols, commonly practiced in clinics, should allow for tolerant cells to eventually take over the population.

Tolerance mutations may be caused by the antibiotic treatment itself, or arise spontaneously. Either way, the small number of tolerant mutants would stay hidden and undetected in the population under normal growth conditions, but survives better when the antibiotic is present and hence would be able to take over the population during the course of repetitive antibiotic treatments. This effect could be predicted by the mathematical model of persistence described by Balaban et al. (2004) and Gefen and Balaban (2009), if one extended it to include a high-persistence mutant, whereby the mutant possesses a higher conversion rate to persisters, but is otherwise

identical to the wild-type (**Supplementary Figure 1a**). This mutant, by itself, would have a tolerant phenotype due to a higher fraction of persisters. If this mutant is mixed with a wild-type strain and subjected to intermittent antibiotic treatments, the resulting population dynamics can be simulated in an evolutionary model, with alternating periods of killing and regrowth (Van den Bergh et al., 2016; Sulaiman and Lam, 2020a; **Supplementary Figure 1b**). In this scenario, the simulation showed that the mixed population would indeed attain a higher and higher survival rate against the antibiotic after several cycles, which can be traced to the invasion of the small subpopulation of the tolerant mutant. Namely, since the mutant has a higher propensity to convert to persister cells, after prolonged treatments, the remaining survivors would have a higher proportion of the mutant. When this residual population is allowed to regrow, the mutant will comprise a higher proportion of the cells than in the previous cycle.

TOLERANCE AND RESISTANCE MUTATIONS IDENTIFIED FROM LABORATORY EVOLUTION EXPERIMENTS

We summarize the tolerance and resistance mutations identified from recent *in vitro* laboratory evolution experiments in **Table 1**. The mechanism of tolerance in the evolved populations appears to be dependent on the treatment conditions, including the bacterial growth phase at which the antibiotic is applied, the type of antibiotic used, and the durations of treatments. For example, overnight cultures of *E. coli* populations repeatedly diluted in a medium containing ampicillin eventually developed high tolerance by increasing their lag time (Fridman et al., 2014). Interestingly, the populations could match the lag time to the duration of antibiotic exposure, or in other words, optimizing their lag time depending on how they have been treated previously through unique mutations. A total of eight mutations were detected across all of their evolved strains. Reversion of three of these mutations restored the lag time of the ancestral strain. The mutated genes are in *vapB*, coding for an antitoxin of the *vapBC* TA module, *metG*, expressing methionyl-tRNA synthetase, and *prsA*, expressing ribose-phosphate diphosphokinase. The first two genes are involved in cellular components (TA modules and aminoacyl-transfer RNA synthetases, respectively) previously implicated in increased persistence (Gerdes and Maisonneuve, 2012; Germain et al., 2013; Kaspy et al., 2013). Although how exactly these mutations led to the extension in lag time is still unknown, quantitative analysis revealed that TA modules might act in a network manner to set the timescale of the lag-time distribution through regulation of the frequency and duration of growth arrest (Rotem et al., 2010). A follow-up study by the same group also revealed that prolonging the treatment cycles eventually caused the tolerant strains to attain resistance through mutations in the promoter of *ampC,* coding for a beta-lactamase associated with ampicillin resistance (Levin-Reisman et al., 2017). Another group that performed laboratory evolution experiments by treating stationary-phase *E. coli* with an aminoglycoside identified a different set of tolerance mutations, located in the genes *oppB, nuoN*, and *gadC*, expressing oligopeptide transport system permease protein, NADH-quinone oxidoreductase subunit N, and glutamate/gamma-aminobutyrate transporter, respectively (Van den Bergh et al., 2016). Their evolved strains showed no extension in lag time, and the tolerance phenotype could not be traced to a reduction in membrane potential (limiting antibiotic uptake) or translation activity (limiting target activity). Competition experiments in the presence of the antibiotic showed that the tolerant strains had a 160 to 360-folds fitness advantage compared to the ancestral strain over a single round of antibiotic treatment, which was primarily caused by a ∼1,000-folds increase in the rate of persister formation during the early stationary phase. Competition experiments in the absence of the antibiotic, on the other hand, showed that the mutants had reduced fitness compared to the ancestor, and the cost was caused by growth deficits linked to the increased proportion of persister cells. When regrown daily in the absence of antibiotic, the tolerant strains eventually reduced their tolerance to a level similar to the wild-type, due to additional compensatory mutations that increase fitness, rather than genetic reversion of the tolerance mutations.

In another study where exponential *E. coli* cells were repetitively treated with different classes of antibiotics (ampicillin, ciprofloxacin, and apramycin), the single point mutations detected from the three evolved populations were completely different, and their proteome profiles were also markedly divergent (Sulaiman and Lam, 2020a). Among the mutated genes, several have been previously linked to persistence and tolerance. For instance, the population repetitively treated with ampicillin bore a mutation in the *cyaA* gene, coding for adenylate cyclase. This enzyme is responsible for the formation of cyclic AMP (cAMP), which regulates genes involved in carbon catabolism, virulence, biofilm formation, and SOS response. It has also been reported that Δ*cyaA* mutants possess increased tolerance to β-lactams through the activation of oxidative stress responses and SOS-dependent DNA repair (Molina-Quiroz et al., 2018). On the other hand, the population treated with ciprofloxacin gained a mutation in *mdoH*, coding for glucosyltransferase H, an enzyme involved in the biosynthesis of periplasmic glucans. This protein was known to be important for biofilm-associated resistance of *Pseudomonas aeruginosa*, where the periplasmic glucans interact physically with antibiotics and prevent them from reaching their sites of action by sequestering the antibiotics in the periplasm (Mah et al., 2003). Lastly, the population from apramycin treatments had a mutation in *fusA*, coding for elongation factor G that catalyzes the ribosomal translocation step during translation elongation. It is also involved in ribosome assembly and recycling, and acts as a catalyst for the interconversion of (p)ppGpp, which plays a role in bacterial persistence. By cross-comparing the regulated proteomes of the three evolved populations generated from different antibiotic treatments, they identified protein candidates with similar expression profiles that might be important for

TABLE 1 | List of mutations identified from recent *in vitro* laboratory evolution experiments.

Study	Name of strain	Gene having mutations*	Phenotype	Phase of growth during treatment	Treatment duration (hours)	Antibiotic used during evolution
Escherichia coli						
Fridman et al., 2014	Tbl3a	*vapB*	Tolerance	Diluted Stationary phase	3	Ampicillin
	Tbl3b	*prsA*			3	
	Tbl5a	*metG, sspA*			5	
	Tbl5b	*vapB, pgm, yeaI*			5	
	Tbl8a	*prsA*			8	
Levin-Reisman et al., 2017	MGYE7-TOL	*prsA*	Tolerance	Diluted Stationary phase	4.5	Ampicillin
	MGYE7-TOLRES	*prsA, ampC*	Tolerance + Resistance			
	KLYE1-TOL	*metG*	Tolerance			
	KLYE1-TOLRES	*metG, ampC*	Tolerance + Resistance			
	EPECE7-TOL	*metG*	Tolerance			
	EPECE7-TOLRES	*metG, ampC*	Tolerance + Resistance			
Van den Bergh et al., 2016	Clone 1-1	*oppB*	Tolerance	Stationary phase	5	Amikacin
	Clone 2-1	*nuoN*				
	Clone 6-1	*gadC*				
Sulaiman and Lam, 2020a,b	Evo3A	*ybbA, yhgE, cyaA*	Tolerance	Exponential phase	3	Ampicillin
Sulaiman and Lam, 2020a	Evo3C	*mdoH, icd*				Ciprofloxacin
	Evo3P	*narZ, fusA*				Apramycin
Khare and Tavazoie, 2020	AC1	*leuS, murP*	Tolerance	Exponential phase	4	Ampicillin + Ciprofloxacin
	AC2	*selU, metG*				
	AC3	*yhjJ*				
	AC4	*ykgJ/ecpE, ribE, pth, yecD, cyaA*				
	AC5	*yecD, metG*				
	AC6	*ileS, ykgJ/ecpE, yecD*				
	AC7	*ykgJ/ecpE, ynfE, yecD, metG*				
	AC8	*proS, ykgJ/ecpE, yecD, yedR/yedS, yfcI*				
	AK1	*ompC, gltP*				Ampicillin + Kanamycin
	AK2	*pth*				
	AK3	*clpX/lon, pth*				
Staphylococcus aureus						
Mechler et al., 2015	D6	*pitA, gltS*	Tolerance	Stationary phase	7.5	Daptomycin

**The mutations shown include single point mutations, insertions and deletions. The exact location of the mutation may not be the same in different strains, although the gene where the mutation occurs is the same. For example, Tbl3b and Tbl8a both have single point mutations in the prsA gene, but the amino acid substitutions are R79H and C60Y in Tbl3b and Tbl8a, respectively.*

tolerance. These include GrcA, a glycyl radical cofactor that have increased expression following the induction of toxin MazF; RaiA and RRF (ribosome recycling factor) which are related to ribosomal activity; AhpF, which protects the cell against DNA damage by alkyl hydroperoxides; NuoF, which plays a role in the electron transport chain; and CysP, a part of the ABC transporter complex. In a newer study where two antibiotics with orthogonal modes of action were used to repetitively treat exponential-phase *E. coli*, the time taken for the cells to develop tolerance is much longer compared to those trained with a single drug. The mutations observed in the tolerant populations were enriched in translation-related genes (*ileS, leuS, metG, proS*, and *pth*) (Khare and Tavazoie, 2020). Through transcriptional profiling, the authors identified overlapping pathways that were

differentially regulated in the evolved strains compared to the wild-type. Gene ontology analysis revealed increased cellular stress in these persister-enriched populations, characterized by the up-regulation of the SOS response and phage shock genes, and the down-regulation of genes involved in core processes such as ATP production, electron transport chain, translation, cell division, and protein transport.

The tolerance mutations detected in laboratory evolution experiments across different laboratories are different and seemingly unrelated, perhaps due to the slight variations in the experimental conditions, the different ancestral strains used for the evolution experiments (with slight mutational variations), and/or the random nature of mutagenesis (Sulaiman and Lam, 2019). The mutated genes were involved in many and varied cellular functions. Evidently, tolerance can be enhanced by a large number of genetic changes throughout the genome, implying that many evolutionary pathways exist for the development of tolerance (Balaban and Liu, 2019). This is in contrast to resistance, which tends to arise due to the alterations in a few well-defined genes directly related to the action mechanism of the antibiotic. Moreover, the diversity of tolerance-associated genes discovered thus far suggested that tolerance may be better thought of the result of a perturbed biological network, and cannot be easily understood from a reductionist point-of-view, again in contrast to resistance. It is therefore not surprising that recent discoveries in the aforementioned laboratory evolution experiments have not led to a well-defined general mechanism for tolerance. Nonetheless, the two recent studies employing system-wide gene expression profiling by transcriptomics (Khare and Tavazoie, 2020) and proteomics (Sulaiman and Lam, 2020a) and cross-comparison of multiple tolerant mutants offer some hope that there might exist some common pathway(s) that underlie tolerance of various types, although much remains to be explored and clarified.

ANTIBIOTIC TOLERANCE BOOSTS THE EVOLUTION OF RESISTANCE

There has been a debate whether antibiotic tolerance and resistance are two distinct phenomena or whether they have any connection at all. Recently, it has become clear that tolerance often precedes resistance in the course of evolution because tolerance mutations occur more frequently than resistance mutations, owing to the larger target size. When the treatment cycles of the evolution experiment were prolonged, Levin-Reisman and colleagues observed that tolerant *E. coli* populations eventually became resistant to the drug (Levin-Reisman et al., 2017). More alarmingly, they also showed that the tolerance mutations facilitated the development of resistance in the populations, and a positive epistatic interaction occurs between the tolerance and resistance mutations (Levin-Reisman et al., 2019). As the authors explained, the probability for the establishment of a mutation in cyclic antibiotic treatment depends on two main factors: the probability of mutation occurrence, and the probability that the mutation is not lost during antibiotic exposure. Tolerance increases the probability of the establishment of resistance mutations in two ways. It supports the continued survival of the population and hence extends the window of opportunity for rarer mutations to occur. At the same time, tolerance also increases the number of survivors, and therefore lowers the probability of resistance mutations getting lost during antibiotic exposure.

Other studies also corroborate the idea that antibiotic tolerance increases the chances for resistance mutations to develop. It was observed that the resistant mutants of *Mycobacterium tuberculosis* came from persisters (Sebastian et al., 2017), which are antibiotic tolerant. Persister cells in natural and laboratory *E. coli* strains have increased mutation rates which should promote the development of resistance (Windels et al., 2019a). Besides, a positive correlation between the number of persisters and the rate of resistance mutations was also noticed in *P. aeruginosa* (Vogwill et al., 2016). These observations of tolerance serving as a driver for resistance development seem to be general in a wide range of experimental setups and bacterial strains. Moreover, the high number of survivors in the tolerant populations, combined with the higher mutation rates due to the increased stress response in these tolerant cells, may act synergistically to increase the likelihood of the occurrence of resistance mutations (Windels et al., 2019b). Apart from genetic mutations in the bacterial chromosome (vertical transmission), resistance can also be acquired through the horizontal gene transfer of genetic elements. The high number of survivors of the tolerant mutant may serve as a reservoir to store the plasmids that would later be passed on to other cells when the antibiotic concentration has dropped. This was shown in a study where *Salmonella* persisters facilitated the spread of antibiotic resistance plasmids (Bakkeren et al., 2019). To make things worse, *in vivo*, these tolerant populations often remain in the host tissues in a dormant, non-growing state (such as in the case of *M. tuberculosis*), and may form biofilms that help them survive the antibiotic attack (Lewis, 2008).

CLINICAL DETECTION OF TOLERANT STRAINS TO PREVENT THE DEVELOPMENT OF RESISTANCE

The typical process of tolerance and resistance development during laboratory evolution experiment is depicted in **Figure 1**. To a certain extent, this resembles the antibiotic therapy commonly adopted in clinics. While the tolerance level of the population (marked by an increase in MDK) keeps increasing over the treatment cycles, the MIC of the population stays the same. However, after the population has attained mutations that confer tolerance, it will greatly speed up the development of resistance and eventually cause the drug to be ineffective. Undetected tolerance is a bane to clinical practice, not only because the surviving cells can regrow and cause the relapse of diseases, but also because it facilitates the development of resistance. Unfortunately, the current standard in clinical practice is focused on screening for resistance through MIC testing. However, tolerance, which is not associated with an increased MIC, is overlooked. Based on the recent findings

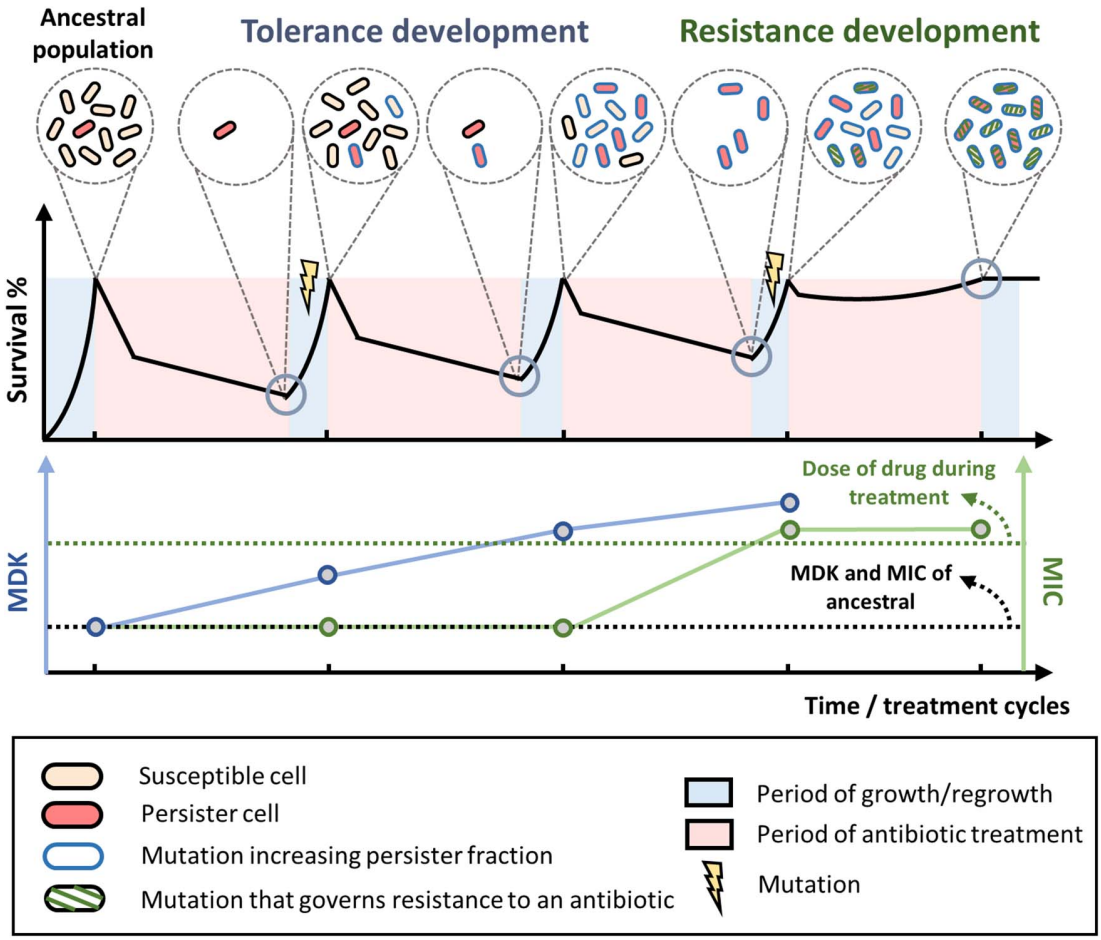

FIGURE 1 | Tolerance and resistance development during antibiotic treatment and regrowth. Typical evolution of bacterial populations under repetitive antibiotic treatments. When treated with high doses of bactericidal antibiotics, biphasic killing is observed where the susceptible cells die rapidly while the persisters survive prolonged treatments. After a few treatment and regrowth cycles, mutations that increase tolerance or persistence fraction may occur (blue outline on the cells), leading to higher overall survival of the population and a higher minimum duration of killing (blue line) toward the antibiotic. Over more treatment cycles, the bacteria may eventually attain mutations that govern resistance to the antibiotic (green diagonal stripes on the cells). Once the MIC of the population (green line) exceeds the dose of the antibiotic used during the treatment (green dotted line), the population can grow within the treatment regime and the antibiotic is no longer effective. MIC, minimum inhibitory concentration. MDK, minimum duration of killing.

described here, it is evident that tolerance should also be screened to reduce the rate of resistance development. For patients who are receiving antibiotic therapy, the tolerance level of the pathogen should be monitored throughout the treatment period. Changing the drug or the treatment condition might be necessary once tolerance has been detected. Since tolerance mutations can be specific to the antibiotic used to treat the cells, switching the drug in time could have a positive impact on clinical outcomes. For example, it has been shown that the tolerance mutations in an evolved *E. coli* population from repetitive ampicillin treatment caused the cells to filament extensively during the treatment, thereby evading ampicillin targets (Sulaiman and Lam, 2020b). The single point mutations on the evolved population led to a perturbed biological network, which then activated the SOS response and suppressed the ROS generation in the cells, triggering filamentation. When treated with other antibiotics, the cells did not filament and the population died off. This highlights the importance for tolerance detection, as the tolerant population may readily be killed with another antibiotic, whereas continued treatment with the same antibiotic is not only ineffective but also dangerous because it promotes the development of resistance.

In another recent study of a patient with *methicillin-resistant S. aureus* (MRSA) bacteremia who received suppressive drug combination treatment, it was shown that resistance development is promoted when the bacteria has attained drug tolerance (Liu et al., 2020). The drug combination (daptomycin and rifampin) was shown to be effective in suppressing resistance development in the MRSA isolate when the cells were still sensitive to the drug. However, once the cells gained tolerance to daptomycin, the drug combination actually increased the chance for rifampin resistance to emerge. In other words, some drug combinations may be effective in preventing resistance development, but it needs to be applied before the cells develop tolerance. This again points to the importance of diagnostic

tools for bacterial tolerance, which will help clinicians to devise suitable therapy that can prevent resistance development. Ideally, differential treatment for susceptible population, population harboring tolerance mutations, and population harboring resistance mutations should become standard practice, as they have distinct survival mechanisms against antibiotic assault.

Fast and easy tolerance detection methods such as TDtest (Gefen et al., 2017) and measurement of MDK_{99} (minimum duration for killing 99% of bacterial cells in the population) (Brauner et al., 2016) can be adopted in clinics. TDtest is a modification of the currently adopted disc diffusion assay for resistance detection. It comprises two steps. The first step is the standard resistance test where the antibiotic disk is applied on the agar plate to determine the inhibition zone. If this were the only step, tolerant cells that survive the transient antibiotic exposure would not be detected due to the lack of nutrients to support visible growth. Therefore, in the second step, the antibiotic disk is replaced by a nutrient disk to compensate for nutrient depletion, thus allowing the detection of tolerant cells, which otherwise would be regarded as susceptible in the standard disc diffusion assay. Another tolerance detection method similar to TDtest is the replica plating tolerance isolation system (REPTIS) (Matsuo et al., 2019). Instead of adding a nutrient disk, colony-forming units (CFUs) on the agar plate containing the antibiotic disk are transferred onto another plate without the antibiotic to allow bacterial growth. Regrowth of bacteria in the zone of inhibition shows the presence of tolerant cells. For rapid detection of tolerance that were caused by an increase in lag time (tolerance by lag), such as those observed in the study of Fridman et al. (2014), automated imaging with ScanLag (Levin-Reisman et al., 2010) or ColTapp (Bär et al., 2020) can be adopted.

FUTURE DIRECTIONS IN STUDYING EVOLUTION OF BACTERIAL TOLERANCE

Although the evolution of bacterial tolerance through repetitive antibiotic treatments has only been recently explored, we already know that tolerance and resistance can be developed in a much shorter timeframe than we previously thought. After merely 3 to 4 treatment cycles, the tolerance level of the treated population is already much greater compared to the ancestral population (Fridman et al., 2014; Sulaiman and Lam, 2020a). This rapid evolution warrants immediate attention from scientists. There is an urgent need to understand how bacteria could adapt so quickly to diverse treatment conditions, and how minor genetic alterations, in seemingly unrelated genes, can provide them with the means to survive antibiotic treatment. More extensive real-time studies of the evolution process of different bacteria toward different treatment conditions are needed, ideally using "omics" methodology that observes the cellular state at the systems level. For instance, a large-scale and high-throughput laboratory evolution study of different bacterial species toward different types of antibiotics should be conducted to comprehensively map the so-called "tolerome" (Brauner et al., 2016; Levin-Reisman et al., 2017), the collection of genes (and proteins) in which mutations affect the tolerance level of the cells. This will give us more insights into the bacteria's adaptation mechanisms, and quicken the development of diagnostic tools. Going forward, more efforts should also be devoted to *in vivo* studies of this phenomenon, since findings in *in vitro* experiments may not directly translate to the host environment which is more complex and heterogeneous. Although laboratory experiments and theoretical predictions showed that bacterial populations could gain high levels of tolerance after a few cycles of repetitive antibiotic treatments (survival ranging from 10 to 100%), pathogenic isolates from patients after frequent antibiotic treatments often do not reach the predicted tolerance levels. It may be because the present models fail to capture some of the "hidden" costs associated with tolerance in the hostile environment of the host (Van den Bergh et al., 2017). Additional factors such as host defense and species competition may also come into play *in vivo* (Sakoulas et al., 2017; Sulaiman and Lam, 2019). Although models for *in vivo* evolution are still lacking, researchers have been performing longitudinal studies of bacteria strains isolated from patients, thereby revealing the dynamics of tolerance evolution within the host (Liu et al., 2020). Such studies are highly valuable. In addition, for patients with severe and recalcitrant infections, combinatorial treatment is often employed (Liu et al., 2020), while most of the reported *in vitro* evolution experiments were limited to a single drug. A laboratory evolution experiment that used drug combination to treat *E. coli* populations showed that longer treatment cycles are required for the populations to finally achieve tolerance, suggesting different evolutionary dynamics (Khare and Tavazoie, 2020). Future laboratory evolution experiments should take into account the use of drug combinations to better simulate clinical conditions, which in theory should be more complicated as drug combinations could act in a suppressive or synergistic manner.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.617412/full#supplementary-material

Supplementary Figure 1 | Evolutionary model simulates the invasion of small number of tolerant mutants after repetitive antibiotic treatment. **(a)** Mathematical

equations of the evolutionary model, which is an extension of the model of persistence. Unlike the model of persistence that assumes two subpopulations, there are four subpopulations in the evolutionary model; wild-type normal population (n_W (t)), wild-type persisters population (p_W (t)), mutant normal population (n_M (t)), and mutant persisters population (p_M (t)). **(b)** The evolutionary model was used to simulate typical laboratory evolution experiments. After a few cycles of antibiotic treatment, the population will have increased survival to the antibiotic. The shaded regions are the period of high-dose antibiotic treatment. The right figure shows that the number of persisters is very low in the ancestral population, which is susceptible to the antibiotic. However, after a few cycles, the small number of tolerant mutants gradually take over the population, which in the end becomes tolerant.

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Quinoline Antimalarials Increase the Antibacterial Activity of Ampicillin

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Bacterial and malaria co-infections are common in malaria endemic countries and thus necessitate co-administration of antibiotics and antimalarials. There have long been anecdotal clinical reports of interactions between penicillins and antimalarial agents, but the nature and mechanisms of these interactions remain to be investigated. In this study, we employed antimicrobial interaction testing methods to study the effect of two antimalarials on the antibacterial activity of ampicillin *in vitro.* Paper strip diffusion, a modified disc diffusion and checkerboard methods were used to determine the nature of interactions between ampicillin and quinoline antimalarials, chloroquine and quinine, against Gram-positive and Gram-negative bacteria. The impact of antimalarials and ampicillin-antimalarial drug combinations on cell integrity of test bacteria were determined by measuring potassium release. The tested antimalarials did not show substantial antibacterial activity but quinine was bactericidal at high concentrations. Chloroquine and quinine increased ampicillin activity, with increasing concentrations extending the antibacterial's inhibition zones by 2.7-4.4 mm and from 1.1 to over 60 mm, respectively. Observed interactions were largely additive with Fractional Inhibitory Concentration Indices of >0.5-1 for all ampicillin-antimalarial combinations. Quinine and, to a lesser extent, chloroquine increase the activity of ampicillin and potentially other β-lactams, which has implications for combined clinical use.

Keywords: penicillins, chloroquine, quinine, ampicillin, drug combination, paper strip diffusion, modified disc diffusion checkerboard

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INTRODUCTION

The nature of interactions between antimalarials, particularly those belonging to the quinoline class, and antibiotics has been studied extensively in the past two decades (Babalola et al., 2002, 2003, 2009; Abreu et al., 2014; Falade et al., 2016). Malaria is immunosuppressive. As a result, patients with malaria often come down with other infections (Morakote and Justus, 1988; Babalola et al., 2003; Pradhan and Ghosh, 2013; Falade et al., 2016; Popoola et al., 2019). Irrespective of how common actual co-infections are, *Plasmodium* and bacterial co-infections are often presumed, resulting in very common co-administration of these two classes of drugs in sub-Saharan Africa where malaria is endemic (Falade et al., 2016; Popoola et al., 2019). For instance, a prescription survey conducted in a tertiary institution in Nigeria showed that antimalarials were the most

commonly prescribed drugs and that half of the patients on antimalarials were also placed on antibiotics (Uchefunah, 2007).

Co-administration of two or more drugs is considered rational when trying to achieve a desired therapeutic objective or treat co-morbidities but the possibility of drug-drug interactions could offset these benefits by bringing about sub-therapeutic drug concentrations that could ultimately lead to treatment failure (Martinbiancho et al., 2007). For instance, penicillin antibiotics have been reported to demonstrate *in vivo* and *in vitro* interactions with certain antimalarial agents (Babalola et al., 2003, 2009; Falade et al., 2016). Documented interactions include reduction in bioavailability of penicillins (ampicillin and cloxacillin) by 40-70 % after oral co-administration with quinine and chloroquine in healthy patients (Ali, 1985; Babalola et al., 2003; Falade et al., 2016). Other studies reported similar interactions with proguanil and artesunate (Babalola et al., 2002, 2009), suggesting that pharmacokinetic drug interaction is likely occurring at the absorption phase (Palleria et al., 2013).

The quinoline antimalarials have longed been used in the treatment of malaria, especially as caused by *Plasmodium falciparum* (Ugale et al., 2017; Center for Disease Control and Prevention [CDC], 2019). Although chloroquine and quinine have been largely phased out from current malaria treatment guidelines, they are still recommended for use in some circumstances. For instance, quinine is a second-line agent in managing complicated malaria and is recommend in pregnant women in their first trimester (World Health Organization, 2015). Chloroquine is recommended by the CDC for the treatment of uncomplicated malaria, and in pregnancy, especially in the first trimester in regions without chloroquine-resistant strains such as Central America west of the Panama Canal, Haiti, the Dominican Republic, and most of the Middle East (Center for Disease Control and Prevention [CDC], 2019). Chloroquine remains an effective choice for most *P. vivax* and *P. ovale* infections. The drug has also been used off-label and in clinical trials to manage SARS-CoV-2 infections, which sometimes require administration of an antibacterial for secondary bacterial infection (Colson et al., 2020; Devaux et al., 2020; Zhonghua Jiehe He Huxi Zazhi, 2020). Furthermore, these old antimalarials have future potential as resurgence in parasites sensitive to chloroquine has been reported in some countries where partial resistance to artemisinin and partner drug resistance exist. Older antimalarials may therefore be an interim solution to antimalarial therapy prior to discovery of newer ones, for instance, cessation of chloroquine use in Malawi was followed by the re-emergence of chloroquine-susceptible malaria (Kublin et al., 2003; Frosch et al., 2011; World Health Organization, 2018).

Of more interest is the fact that chloroquine and quinine have been reported to have some antibacterial activity, albeit at high concentrations, arising from their structural similarities to (Wolf et al., 2002; Lv et al., 2007; Davidson et al., 2008; Kharal et al., 2009; Bawa et al., 2010; Achan et al., 2011; Jagadeesh et al., 2014) quinolone antibacterials.

This study attempts to clarify the nature of interactions between penicillins and quinoline antimalarials using a range of testing methods. This investigation is long overdue since the drugs in question have been in clinical use for more than half a century, often in combination. We evaluated, *in vitro*, the effect of chloroquine and quinine on the antibacterial effect of ampicillin, against ampicillin-sensitive and -resistant isolates.

MATERIALS AND METHODS

Cultivation of Strains and Inoculum Preparation

Table 1 shows the test organisms used in the study. Isolates were maintained in Luria Broth: glycerol 1:1 at −80 °C and cultured on Muller Hinton agar (MHA; Oxoid, United Kingdom) at 37°C overnight prior to use. To prepare bacterial suspensions, three morphologically similar colonies from each respective agar plate were suspended in 4 mL of 0.9 %w/v saline (BDH Chemical LTD, Poole England) and standardized by adjusting to 0.5 McFarland Standard to produce final inocula of 1–5 × 10^8 CFU/mL according to the Clinical and Laboratory Standards Institute (CLSI-M07A11) guidelines (Clinical and Laboratory Standards Institute [CLSI], 2018).

Test Compounds

Commercially procured powders of quinine sulfate (Sigma-Aldrich, United Kingdom), chloroquine phosphate (Sigma-Aldrich, United Kingdom), ampicillin sodium (Merck, United Kingdom), cloxacillin sodium monohydrate (Sigma-Aldrich, Germany), and nalidixic acid (Merck, United Kingdom) were used in the study. Test antibiotic solutions were prepared as outlined in the Clinical and Laboratory Standards Institute (CLSI) guideline. All test compounds were dissolved in water except nalidixic acid which was dissolved in 0.1 N sodium hydroxide (O'Neil, 2001). Stock solutions were prepared at 10 mg/mL for quinine and chloroquine and 1 mg/mL for ampicillin, cloxacillin, and nalidixic acid (Wiegand et al., 2008; Clinical and Laboratory Standards Institute [CLSI], 2018). Fresh

TABLE 1 | Organisms used in the study.

Strain	Species	Relevant properties	Reference or Source
ATCC 25922	*Escherichia coli*	Sensitive to ampicillin: CLSI-recommended control organism for antimicrobial susceptibility testing	Selectrol, TCS Biosciences, United Kingdom.
LLH029E	*Escherichia coli*	Ampicillin-resistant	Fecal isolate (Molecular Biology Lab, University of Ibadan)
NCTC 6571	*Staphylococcus aureus*	Sensitive to penicillin, cloxacillin and ampicillin; CLSI-recommended control organism for antimicrobial susceptibility testing	Selectrol, TCS Biosciences, United Kingdom

TABLE 2 | MIC of antibiotics and antimalarials against tested isolates using broth microdilution method ($n = 3$).

Drug tested	MIC and MBC of test drugs (μg/mL)						
	E. coli ATCC 25922		*S. aureus* NCTC 6571		*E. coli* LLH029E		*S. aureus* ATCC 29213
	MIC	MBC	MIC	MBC	MIC	MBC	MIC
Ampicillin	3.1	6.2	0.78	1.56	>100	ND	3.1
Cloxacillin	100	>100	0.19	0.390	>100	>100	NT
Nalidixic acid	4	8	256	512	NT	NT	64
Quinine	1,000	1,000	1,000	1,000	1,000	1,000	NT
Chloroquine	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	NT

ND, means not determined; NT, not tested.

stock solutions of ampicillin, cloxacillin and nalidixic acid was made for each experiment.

Antimicrobial Susceptibility Testing

The antimicrobial activities of chloroquine, quinine and ampicillin were examined by determining the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) against the test organisms using the broth microdilution method as laid out in the CLSI M07-A11 guideline (Clinical and Laboratory Standards Institute [CLSI], 2018). The tests were performed in sterile, polystyrene 96-well round bottomed microtiter plates. Bacterial suspensions standardized by adjusting the turbidity with a spectrophotometer equivalent to a 0.5 McFarland standard (optical density of 0.08–0.13 at 625 nm, at 1-cm light path) were added to the wells of the microtiter plate containing 100 μL of twofold serial dilutions of the test antimicrobial to give final inoculum size of 5×10^5 CFU/mL. Wells without the test organisms served as sterility control while inoculated wells without the drugs served as positive (growth) control. The plates were incubated at 37 °C for 24 h after which they were visually inspected and the OD_{595} was recorded using a microtiter plate reader. The MIC was defined as the lowest concentration of each drug resulting in complete inhibition of growth. The MBC was determined by sub-culturing the wells in the MIC microtiter plate into corresponding wells of a sterile microtiter plate containing 100 μL of Muller Hinton broth using a multi-point inoculator. The plate was then incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration of each drug that inhibited bacterial growth compared to the untreated control culture, as shown by lack of turbidity in the wells. Not more than 2 microtiter plates were stacked in the incubator although CLSI allows up to 4 plates. Concentrations ranging from 7.8125 and 1,000 μg/mL were tested for chloroquine and quinine, and concentrations between 0.0122 and 50 μg/mL were tested for ampicillin and cloxacillin. Assays were performed in triplicates.

Paper Strip Diffusion Test

This method is a qualitative approach to evaluating interactions between two compounds. To make ampicillin paper strips, ampicillin stock solution was prepared at 20 μg/mL in distilled water. Dilution of the stock solution was done to make a working solution of 2 μg/mL, from which 1 mL was added to individual 1 mm thick sterile Whatman filter paper strips (0.5 × 4 cm) to make ampicillin strips (2 μg). In a similar manner, chloroquine and quinine stock concentrations at 50 μg/mL were diluted to make 5 μg/mL working solutions, from which 1 mL was added to corresponding strips to make quinine strips (5 μg). When dry (after circa 2 h), the strips were placed adjoining but non-overlapping in a T-conformation on a MHA plate that had been surface-inoculated with a standardized suspension of the test organisms. Drugs were allowed to diffuse from the filter strips into the medium for 30 min at room temperature. The plates were then inverted and incubated at 35 ± 2 °C for 24 h. Plates with filter paper strips that had no drugs in them were used as growth controls. Commercially procured trimethoprim and sulfamethoxazole discs (Oxoid) arranged as strips in the T-conformation was used as a positive control for synergism (Bushby and Hitchings, 1968; Bernstein, 1982) and similarly placed ampicillin and trimethoprim disc combinations were used as a "no-interaction" control. The pattern of growth of test organisms was interpreted as follows: broadening of the zones of inhibition at adjoining ends depicts synergism, depression or narrowing of the zones indicates antagonism while no effect on the zones of inhibition indicate indifference (Laishram et al., 2017).

Modified Disc Diffusion (MDD) Assay

In this method, disc diffusion tests are performed after incorporation of an agent in the agar medium to determine the nature of interactions between the agent in the medium and that in the disc (Amin et al., 2015; de Ruyck et al., 2016; Laishram et al., 2017). Doubling dilutions of the antimalarials (62.5–1,000 μg/mL) were made in molten Muller Hinton agar, which was then poured and set in plates aseptically. Bacteria inocula were standardized by adjusting the turbidity to 0.5 McFarland standard (OD_{625} at 0.08–0.13). The standardized inocula were spread on the agar surface according to the CLSI disc diffusion protocol (Clinical and Laboratory Standards Institute [CLSI], 2018) and ampicillin discs (10 μg) were applied. Mueller Hinton agar with no antimalarial drug was used as control. The plates were left at room temperature for about 1 h to allow diffusion of the antibiotic in the disc into the agar and then inverted and incubated at 35 ± 2 °C for 16–20 h. Diameters of zone of inhibition in millimeters were measured and plotted against log of chloroquine and quinine concentrations. In comparison with

the inhibition zone of the ampicillin-only tests, an increase in bacterial zone diameter of ≥2 mm in the ampicillin-antimalarial containing disc is defined as synergy, an increase of <2 mm is considered weak synergy while a reduction in inhibition zone is defined as antagonism (Amin et al., 2015; Laishram et al., 2017). Assays were done in triplicate and repeated at least three independent times.

Checkerboard Assay

The interactions between ampicillin and itself, chloroquine, quinine and nalidixic acid, and between cloxacillin and chloroquine and quinine against a selection of the test strains were studied using the checkerboard technique. To test, identical concentrations of ampicillin at 16 times its MIC, were added to the first well of each row and diluted twofold along the columns of a 96-well round- bottom microtiter plate (Greiner Bio-One, Germany). In a similar manner, concentrations of the antimalarial added to the top well in a column were diluted along the rows to give serial twofold dilutions. The two doubling dilutions were combined to yield a checkerboard with control wells on the lowest row and rightmost columns. Wells were challenged with the standardized inoculum, except for the sterility control wells, and the plates were incubated at 37 °C for 24 h. All tests were performed in triplicate. Optical density (OD_{595}) readings were taken and the nature of interaction of the drug combinations were classified on the basis of the fractional inhibitory concentration index (FICI), i.e., the combination of ampicillin-antimalarial that produced the greatest change from ampicillin alone. The FICI is calculated for each strain and drug combination using the formula:

$$FIC\ index\ (FICI) = FICA + FICB$$

Where

FICA = MIC of drug A in the combination/MIC of drug A alone.

FICB = MIC of drug B in the combination/MIC of drug B alone.

Interactions were interpreted as synergistic if the FICI ≤ 0.5, additive if the FICI is > 0.5–1, indifferent if FICI > 1 < 2 and antagonistic if the FICI ≥ 2 (European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), 2000); Amin et al., 2015).

Effect of Ampicillin-Antimalarial Combinations on Bacterial Cell Membrane Integrity

The leakage of potassium from the cells of test organisms was used to evaluate loss of membrane integrity caused by the ampicillin alone and when combined with quinine and chloroquine. The drug combination concentrations that led to a broadened inhibition zone in the disc diffusion assay was used. The test bacterial cells were grown in nutrient agar at 37 °C for 18 h and centrifuged at 3,700 rpm for 15 min. The cells were washed three times with 0.9 %w/v saline, re-suspended in 20 mL of the normal saline and challenged with individual drug solutions and drug combinations. The resulting solution was placed in an incubator-shaker at 37 °C for 24 h. After that, the cellular debris were separated by centrifugation at 3,500 rpm for 15 min and the supernatant was filtered through a 0.45 μm membrane filter. Aliquots of the supernatants were taken and stored in sample bottles which were frozen at −80 °C. The presence of potassium ions present in the samples were carried out using a flame photometer (PFP7, Jenway, Sweden) at wavelength of 766.480 nm. The instrument was calibrated using standard solutions containing 1, 5, 10, 15, and 20 μg/mL potassium chloride solutions. Chlorocresol was used as positive control while an inoculum suspension not treated with test compounds served as negative control. In all assays, the leakage of cellular components from bacteria into normal saline (blank) was subtracted from all samples.

Statistical Analysis

Mean and standard deviations of replicates were summarized using Microsoft Excel while correlation analysis was used to find the relationship between drug concentration and inhibition zones at 5% level of significance. All variables represent mean values of three replicates.

RESULTS

Quinine Possesses Antibacterial Activity and Both Chloroquine and Quinine Increase Antibacterial Activity of Ampicillin

Chloroquine did not demonstrate detectable antibacterial activity against either of the type cultures (MIC > 1,000 μg/mL) although decreasing turbidity was observed in the wells with increasing concentrations of chloroquine (**Table 2**). At chloroquine concentrations higher than 1,000 μg/mL, the drug's solubility was poor. Quinine had MIC values of 1,000 μg/mL against *Escherichia* coli ATCC 25922 and *Staphylococcus aureus* NCTC 6571. We recorded MICs of 3.1 and 0.78 μg/mL for ampicillin against *E. coli* ATCC 25922 and *S. aureus* NCTC 6571, respectively. Cloxacillin had MICs of 100 and 0.19 μg/mL against *E. coli* ATCC 25922 and *S. aureus* NCTC 6571, respectively, and nalidixic acid had MIC values of 4 and 256 μg/mL against *E. coli* ATCC 25922 and *S. aureus* NCTC 6571, respectively. None of the penicillins tested showed activity against ampicillin-resistant isolate *E. coli* LLH029E (MIC > 100 μg/mL). Quinine inhibited this strain at 1,000 μg/mL.

As shown in **Table 2**, MBCs were twofold greater than respective MICs for all drugs except quinine, which was bactericidal at its MIC of 1,000 μg/mL.

The paper strip diffusion method qualitatively illustrated interactions between the test compounds (**Figure 1**). Potentiation was observed with combinations of ampicillin and chloroquine and ampicillin and quinine: the zones of inhibition around ampicillin protruded vertically toward the strips containing chloroquine and quinine (chloroquine and quinine showed no activity at the concentrations tested). This protrusion was more marked with quinine against *S. aureus* NCTC 6571.

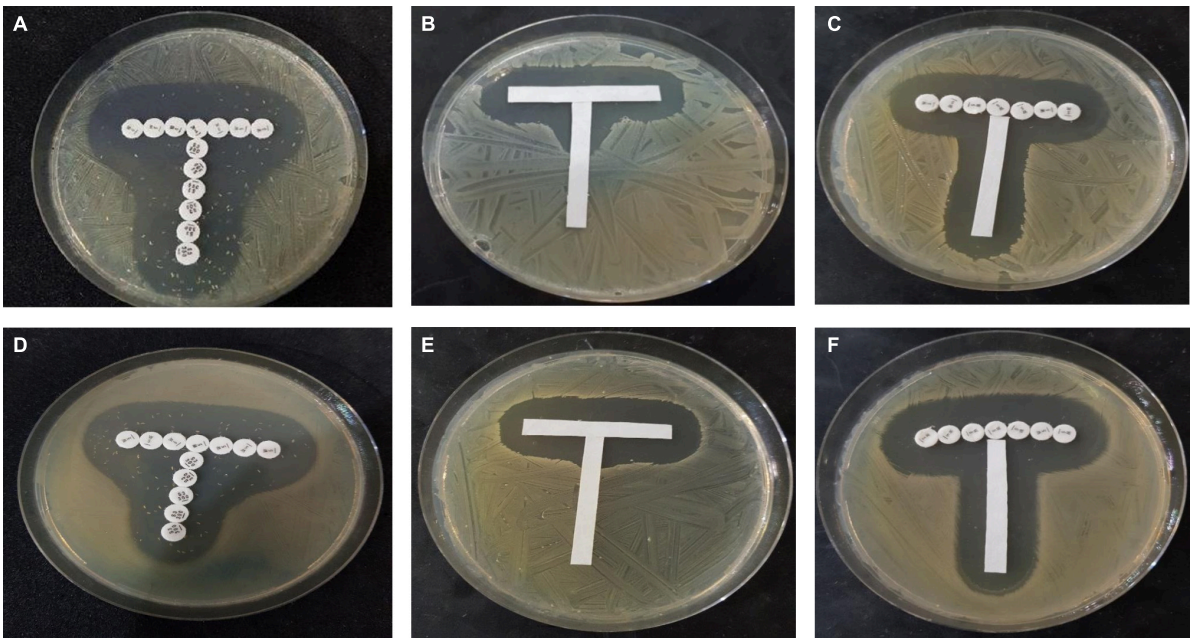

FIGURE 1 | Paper strip diffusion test showing **(A,D)**, synergism between control antimicrobials trimethoprim (horizontally placed) and sulfamethoxazole (vertically placed); **(B,E)** potentiation of ampicillin strip (horizontal) by quinine (vertical); and **(C,F)** no interaction/slight inhibition between trimethoprim (horizontal) and ampicillin (vertical). **(A–C)** Show interaction against *S. aureus* NCTC 25922 while **(D–F)** shows interaction against *E. coli* ATCC 25922.

Broadening of the inhibition zones of both trimethoprim and sulfamethoxazole, indicative of a synergistic effect, was observed with the positive control thus validating the experiment. In the negative control, the ampicillin and trimethoprim had well defined zones of inhibition indicating no interaction between the test compounds, this can be regarded as indifference.

The modified disc diffusion test showed a concentration-dependent increase in the zones of inhibition around ampicillin discs for both quinine and chloroquine (**Figures 2**, **3**). The agar plates containing ampicillin disc only (no antimalarial embedded in the agar medium) had average zones of inhibition (mm) of 15.1 ± 0.1 and 19.2 ± 0.16 around the discs in agar inoculated with *E. coli* ATCC 25922 and *S. aureus* NCTC 6571, respectively. These zone diameter sizes indicate intermediate activity against *E. coli* ATCC 25922 and susceptibility for *S. aureus* NCTC 6571 according to the CLSI guideline (Clinical and Laboratory Standards Institute, 2014). The relationship between the square of the distance d^2 (from the edge of the disc till the edge of the inhibition zone) and the log of the concentration of antimalarial in combination was linear with quinine for *E. coli* ATCC 25922 ($R^2 = 0.9946$, $p = 0.07$) and for *S. aureus* NCTC 6571 ($R^2 = 0.9973$, $p = 0.04$) and exponential with chloroquine for *E. coli* ATCC 25922 ($R^2 = 0.8814$, $p = 0.08$) and for *S. aureus* NCTC 6571 ($R^2 = 0.9444$, $p = 0.01$). No zone diameter was reported for quinine at 1,000 μg/mL because growth was completely inhibited throughout the plate.

We performed a checkerboard experiment to more rigorously describe the ampicillin-quinolone interaction. **Figures 4–6** display the interactions that were seen on a fine-scale, based on turbidity. They show that while the antimalarials did not fully inhibit growth at most of the test concentrations, they did have some inhibitory effects on their own and extended the inhibition of the penicillins. This was easily observed in the checkerboards of the sensitive strains (**Figures 4**, **5**) but was also evident at the highest ampicillin dilution for the ampicillin resistant *E. coli* LLHO29E (**Figure 6**). FICIs were calculated based on absolute inhibition. The MICs in the checkerboard in non-combined wells were consistent with the values obtained from the independent broth dilution method reported in **Table 2**. The concentrations of ampicillin tested ranges from dilutions below and above the MIC but with quinine and chloroquine, the highest concentration tested was the MIC because above these concentrations, the drug did not completely dissolve in water.

The FICI values for ampicillin-quinine combinations were additive based on the interpretative criteria for both *E. coli* ATCC 25922 (FICI = 1.0) and *S. aureus* NCTC 6571 (FICI = 0.75). Additivity was observed with ampicillin-chloroquine combination against *E. coli* ATCC 25922 (FICI = 1.0) and indifference against *S. aureus* NCTC 6571, respectively (FICI = 1.25). *E. coli* LLH029E was fully resistant to both chloroquine and ampicillin and there was growth in all wells containing ampicillin-chloroquine combinations, although, turbidity hence optical density values decreased with increasing concentration of chloroquine (**Figure 6**). Quinine completely inhibited the growth of the *E. coli* LLH029E, an ampicillin resistant isolate, at its MIC but did not make the organism susceptible to ampicillin as growth was observed in wells containing combinations of ampicillin and quinine at concentrations of quinine lower than its MIC. There was, however, decreasing turbidity (and OD_{595} values) in wells as

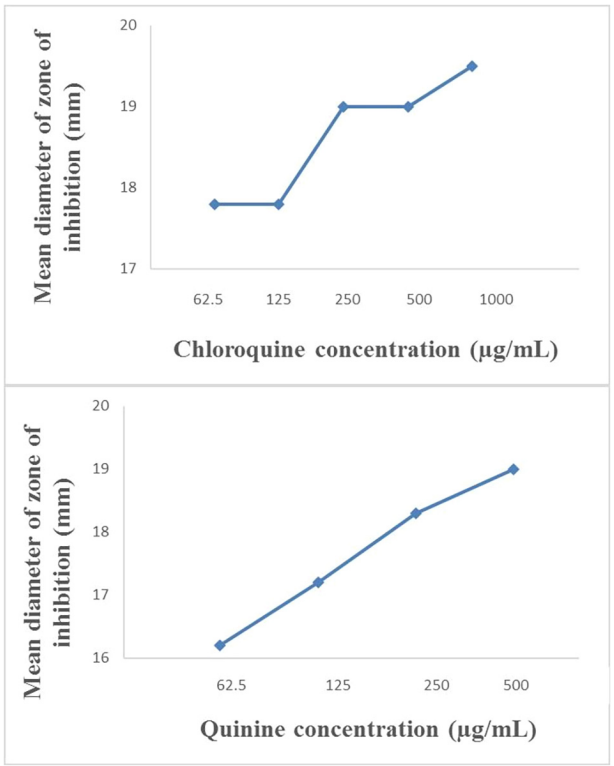

FIGURE 2 | Plot of inhibition zone diameter against log of chloroquine and quinine concentration in combination with ampicillin (10 µg) using *E. coli* ATCC 25922 as test organism.

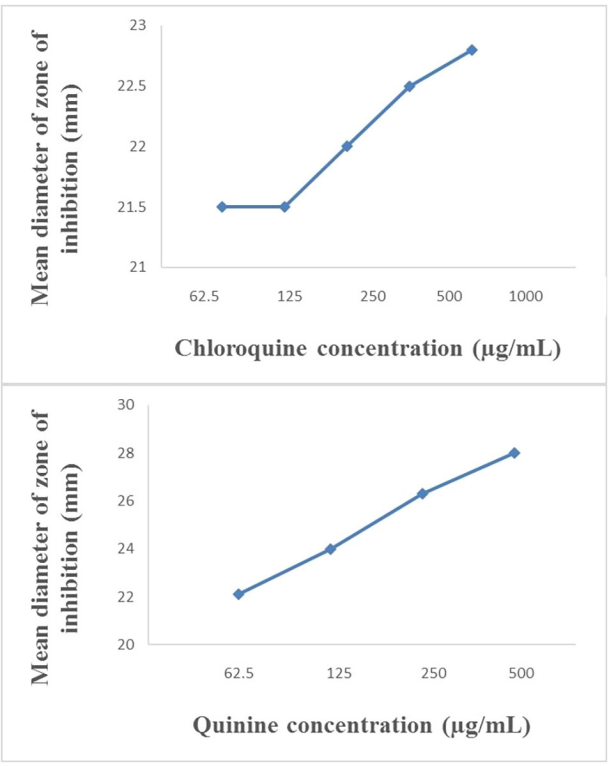

FIGURE 3 | Plot of inhibition zone diameter against log of chloroquine and quinine concentration in combination with ampicillin (10 µg) using *S. aureus* NCTC 6571 as test organism.

the concentration of quinine increased (**Figure 6**). Cloxacillin-quinine and cloxacillin-chloroquine combinations showed additive effects against *S. aureus* NCTC 6571 (FICI = 1.0 and 0.5, respectively). Ampicillin-nalidixic acid combinations showed indifference against *E. coli* ATCC 25922 and *S. aureus* NCTC 6571 (FICI = 1.5). Ampicillin-ampicillin combination was additive against *E. coli* ATCC 25922 (FICI = 0.75) and cloxacillin-cloxacillin combination was additive against *S. aureus* NCTC 6571 (FICI = 0.80) as expected with a drug in combination with equal concentrations of itself (Loewe, 1928).

Observed Increase in Activity of Ampicillin by Antimalarials Is Likely Due to Unfettered Activity at Separate Targets

Since the checkerboard experiment revealed that the interaction between ampicillin and the different antimalarials was additive, the most likely explanation for it is independent activity at their separate targets. We, however, wanted to rule out, or otherwise, any possibility that the antimalarials were either increasing access of β-lactams to their target or directly affecting the activity of the cell wall active antibacterials. Significant potassium ion release was produced by chlorocresol (positive control) with both test organisms. As shown in **Table 3**, potassium ion leakage into the culture media from Gram-positive *S. aureus* NCTC 6571 mediated by ampicillin was not enhanced by the presence of any of the antimalarials. For Gram-negative *E. coli* ATCC 25922, neither ampicillin nor the antimalarials produced significant leakage alone or in combination. All in all, the data rule out a cell-integrity-centered mechanism for the antimalarial-ampicillin interaction.

DISCUSSION

Malaria can cause immune suppression, leaving malaria-burdened patients prone to bacterial infections (O'Dempsey, 2000; Adesanmi et al., 2011; Falade et al., 2016). Clinical co-administration is common in malaria endemic areas, prompting some studies of interactions, which have yielded reports of significant antagonistic drug-drug interactions between penicillin antibiotics and antimalarials when taken concurrently in healthy populations (Ali, 1985; Babalola et al., 2002; Babalola et al., 2003; Babalola et al., 2009; Falade et al., 2016).

Falade et al. (2016) reported an increase in the MIC and MBC of ampicillin and cloxacillin in the presence of quinine against *Staphylococcus aureus* (Falade et al., 2016). This study, however, did not use media recommended for MIC testing, had very wide confidence intervals, did not test for the antibacterial activity of quinine and did not establish a concentration-dependent interaction between the two agents. Thus, while suggested

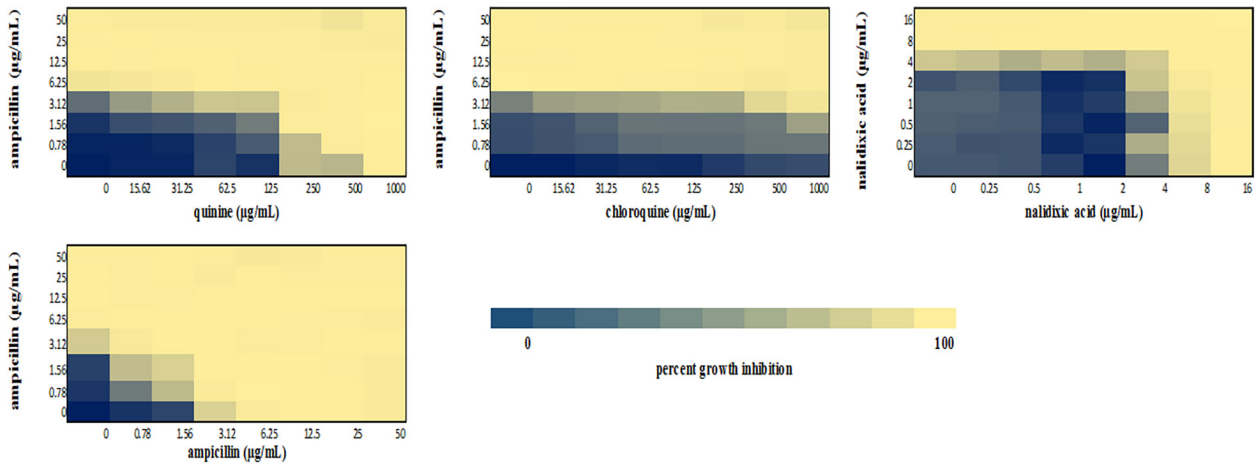

FIGURE 4 | Checkerboard analysis of drug combinations tested against *E. coli* ATCC 25922. Data presented as a heatmap indicating percent growth inhibition based on OD_{595} values. Percent growth reduction values was calculated as 100% – [(OD of treated cells/OD of untreated cells) × 100%] (Ogundeji et al., 2017). Values represent mean values of three replicates.

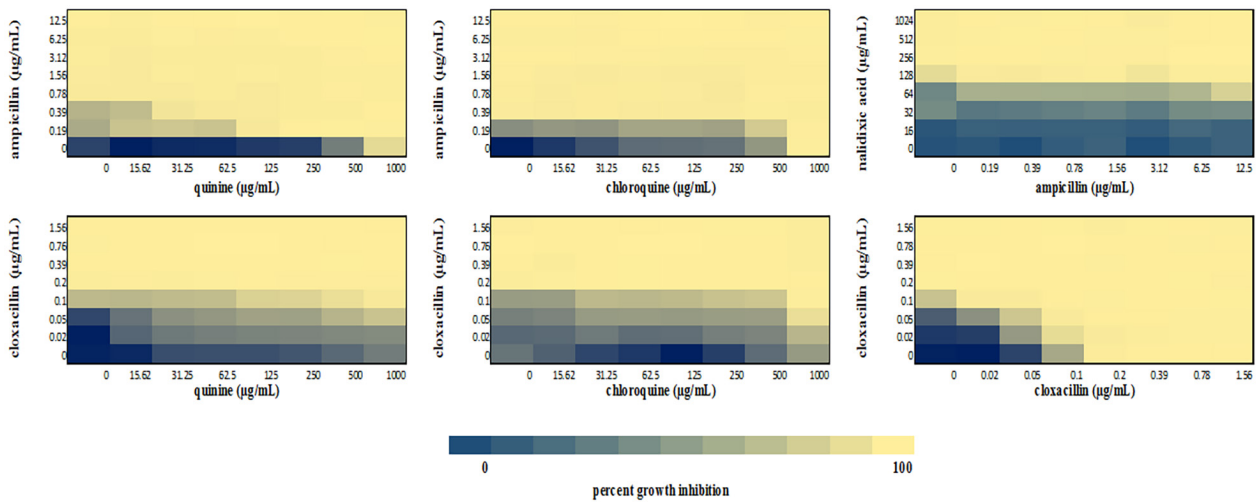

FIGURE 5 | Checkerboard analysis of drug combinations tested against *S. aureus* NCTC 6571. Data presented as a heatmap indicating percent growth inhibition based on OD_{595} values. Percent growth reduction values was calculated as 100% – [(OD of treated cells/OD of untreated cells) × 100%] (Ogundeji et al., 2017). Values represent mean values of three replicates.

mechanisms of pharmacokinetic interactions in the reported *in-vivo* studies are plausible, no published report has adequately described the nature of any interactions or rigorously confirmed the antagonism *in vitro*. In contrast to Falade et al. (2016) one earlier *in vitro* study found no interaction or slight potentiation of ampicillin by quinine (Abreu et al., 2014). For these reasons, a more rigorous exposition of the antibacterial interactions was performed in this study.

The MIC values of ampicillin and nalidixic acid against *E. coli* ATCC 25922 (3.1 and 4 μg/mL, respectively) were consistent with those reported in the literature (Andrews, 2001; Kingdom and Dickinson, 2002; Clinical and Laboratory Standards Institute [CLSI], 2018), as was the MIC of cloxacillin which was below 2 μg/mL (0.19 μg/mL) (Matynia et al., 2005; Al-Harbi et al., 2017; Buldain et al., 2018). The MIC value of 256 μg/mL obtained with nalidixic acid against *S. aureus* NCTC 6571 is consistent with that reported by Andrews (2001) (>128 μg/mL) while the MIC of ampicillin against *S. aureus* NCTC 6571 (0.78 μg/mL) is within the range of 0.06–2 μg/mL reported in literature (European Committee for Antimicrobial Susceptibility Testing [EUCAST], 2000; Andrews, 2001; Adeleke and Olaitan, 2010; Jeyaseeli et al., 2012; Fratini et al., 2017; Rishi et al., 2018).

In this study, quinine showed detectable antibacterial activity against the three organisms used in the study with an MIC of 1,000 μg/mL against *E. coli* ATCC 25922, *S. aureus* NCTC 6571, and *E. coli* LLH029E. The MIC falls within the reported range of values (Kharal et al., 2009; Abreu et al., 2014). Our data show that quinine is bactericidal at 1,000 μg/mL and while

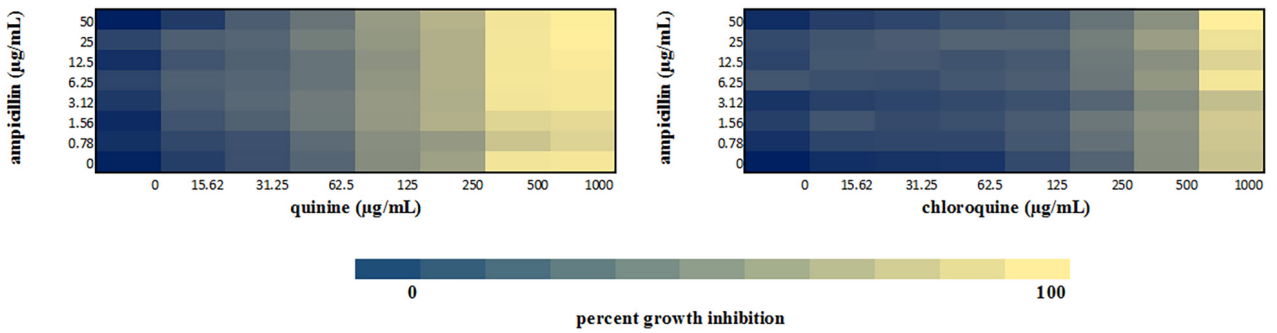

FIGURE 6 | Checkerboard analysis of drug combinations tested against *E. coli* LLHO29E. Data presented as a heatmap indicating percent growth inhibition based on OD_{595} values. Percent growth reduction values was calculated as 100% –[(OD of treated cells/OD of untreated cells) × 100%] (Ogundeji et al., 2017). Values represent mean values of three replicates.

this concentration is unlikely to be achieved physiologically during treatment, the additivity uncovered in this work indicates that lower concentrations may produce therapeutic effects in combination with antibacterials. The same is possibly true for chloroquine for which we could not record an MIC in this study because higher concentration could not be solubilized. We did see decreasing turbidity in wells with increasing concentrations of chloroquine (**Figures 4–6**), indicating some antibacterial properties. Studies have also reported antibacterial activity of chloroquine: an early study reported a pH dependent inhibition of exponential growth of *E. coli* cultures by chloroquine (Wiseman, 1972; Middleton and Wiseman, 1974). Another study reported inhibition zones with chloroquine concentrations as low as 30 μg (Jagadeesh et al., 2014) and more recently, MIC values ranging between 625 and 1,200 μg/mL against susceptible *E. coli* isolates and between 5,000 and 80,000 μg/mL against ciprofloxacin resistant isolates have been reported (Davidson et al., 2008). This is unsurprising since quinolone class of antibacterial drugs were first discovered as by-products from the synthesis of chloroquine (Naeem et al., 2016).

TABLE 3 | Cell permeability assay results (potassium leakage test) of drugs and drug combinations against quality control strains.

Test drug and drug combinations	Potassium release (mg/L)	
	E. coli ATCC 6571	*S. aureus* NCTC 6571
Ampicillin	1.5 ± 0.5	4
Quinine (1,000 μg/mL)	2	2
Chloroquine (1,000 μg/mL)	1.8 ± 0.17	2
Ampicillin + Quinine (125 μg/mL)	2.07 ± 0.81	4.33 ± 0.29
Ampicillin + Quinine (250 μg/mL)	1.67 ± 0.29	4.3
Ampicillin + Quinine (500 μg/mL)	1.33 ± 0.58	3.5 ± 0.5
Ampicillin + Quinine (1,000 μg/mL)	1.17 ± 0.29	3.83 ± 0.29
Ampicillin + Chloroquine (125 μg/mL)	1	4
Ampicillin + Chloroquine (250 μg/mL)	1	3.33 ± 0.58
Ampicillin + Chloroquine (500 μg/mL)	1	3.33 ± 0.58
Ampicillin + Chloroquine (1,000 μg/mL)	1	3.5 ± 0.5
Chlorocresol	5	6.67 ± 0.29

Data are mean ± SD, or mean where SD = 0.

Broadening of inhibition zones around the contact point of the two strips embedded with different agents is often seen in synergism, where both agents are active, or potentiation when one agent is active in the paper strip diffusion test we employed (Lorian and Fodor, 1974; Laishram et al., 2017). Our paper-strip test did indicate that quinine and chloroquine potentiated the activity of ampicillin but as the test is qualitative and has not been widely evaluated, we performed other experiments to study the interactions.

The disk diffusion test modified for testing interactions between drug combinations was first described by Climo et al. (1999) where they determined its validity for uncovering synergy of combinations of vancomycin and beta-lactam antibiotics against staphylococci with reduced susceptibility to vancomycin (Climo et al., 1999). It is now commonly used to study drug interactions (Kiraz et al., 2010; Abreu et al., 2014; Amin et al., 2015; Sy et al., 2016) and the presence of interactions is subject to the method of interpretation. Some interpretations include a weak synergy, defined as a <2 mm increase in zone (Laishram et al., 2017), this definition might fit more appropriately to combinations of ampicillin and quinine on ATCC 25922 where a +1 mm increase in zone diameter was observed if juxtaposed with results of the paper strip assay where potentiation was observed. An increase in inhibition zone by ≥ 2 mm indicates synergism or at the least additivity between ampicillin and quinine on *S. aureus* and at the MIC of quinine, total synergy occurred in both isolates, demonstrated by complete inhibition of growth throughout the entire agar surface. A resulting straight line when the square of zone diameter is plotted against increasing log concentrations of quinine ratifies the interpretation (**Figures 2**, **3**). Combinations of ampicillin and chloroquine against the two isolates may be better than indifferent, since the relationship between the square of inhibition zone sizes and increasing log concentrations was exponential (**Figures 2**, **3**), similar to some previous reports (Jain, 2003; Abreu et al., 2014; Jagadeesh et al., 2014).

The gold standard checkerboard method was lastly used to characterize the type of interaction between ampicillin-quinine, and ampicillin-chloroquine combinations on *E. coli* ATCC 25922, *S. aureus* NCTC 6571, and *E. coli* LLH029E. Since ampicillin is often combined with cloxacillin in dosage forms to reduce resistance against penicillinase-producing Gram-positive bacteria (Martindale, 2011), the interaction between cloxacillin and chloroquine, as well as cloxacillin and quinine was studied in *S. aureus* NCTC 6571 (Moody, 2002; Martindale, 2011; Farrington, 2012). Ampicillin concentration ranges between 16 and 1/4 times the MIC were tested but the highest quinine concentration tested was at the MIC because over this concentration, supersaturation of the drug in its solvent caused the drug to crystallize out obscuring reading of the results. Since chloroquine did not show antibacterial activity indicated by complete inhibition of growth the same concentration range was used for both chloroquine and quinine.

Our checkerboard experiment allowed for simultaneous determination of MIC and FIC on the same microtiter plate and therefore the same dilutions of drugs and test organisms, allowing variations to affect the determination the same way, even among replicates (Fratini et al., 2017). This is often not accounted for in methods that offer more strict interpretive guidelines (White et al., 1996; Odds, 2003). The checkerboard assay results revealed additivity between ampicillin and the quinoline antimalarials tested. This corroborates the results of the MDD assay and paper strip diffusion tests as well as the earlier report of Abreu et al. (2014).

The most logical explanation for additive activity is that each agent exerts its activity without interfering with the mechanism of the other. Because paper-strip and MDD activities suggested that some potentiation or synergism was possible, we sought to determine whether the antimalarials affected membrane integrity or operated in any way at the penicillin target.

Damage to cell membranes, which is often secondary to cell wall disruption, is characterized by discernable leakage of cytoplasmic constituents, especially low molecular weight constituents such as potassium ions (Abbanat et al., 1998; Amarnath et al., 2003; Epstein, 2003; Johnston et al., 2003; Amin et al., 2015; El-Batanony, 2017). Quinine and chloroquine alone, and in combination with ampicillin did not result in appreciable amounts of potassium leakage even at the MIC of quinine (**Table 3**). This suggests that induction of cell leakage is not responsible for the observed additive effects of these antimalarials with ampicillin.

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CONCLUSION

In conclusion, our investigations reveal that chloroquine and quinine have some antibacterial activity and yield at least additive effects at high concentrations when combined with ampicillin and most likely other penicillins (interaction with cloxacillin also yielded additivity) *in vitro*. The data clearly refute antagonism between the two drug classes. The use of chloroquine and quinine in the treatment of malaria may therefore offer an additional advantage of preventing or curing bacterial infections, even against resistant isolates provided that they are not counteracted by interactions at the biopharmaceutic or pharmacokinetic level. Increased antibacterial activity may especially be achievable in non-oral formulations where no antagonistic interactions between antimalarials and penicillins have been reported. For orally administered drugs, it is possible that the additive activity could, at the very least, counterbalance the earlier reported negative effects of quinolone antimalarials on ampicillin pharmacokinetics (Falade et al., 2016). Additivity likely arises from mutual non-interference of antibacterial activity. We have been able to rule out cell leakage as the means by which quinine and chloroquine exert their additive effects with ampicillin. These antimalarials therefore likely exert their additive effects without interacting with the mechanism of antibacterial action of ampicillin. Further *in vivo* investigations are recommended to determine whether this is a subtle synergistic effect, and if so, any mechanism by which this occurs. Lack of interaction between chloroquine /quinine and nalidixic acid combinations suggest that inhibition of DNA gyrase may not be the mechanism of additive interactions and other mechanisms should be investigated. It would also be worth testing other antibacterial-antimalarial combinations, particularly those that are in greater use in today's clinics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

OAO performed literature review, performed, analyzed and interpreted experiments, provided resources, and drafted the manuscript. CB conceived the project, contributed to literature review, co-supervised OAO, interpreted data, and performed critical review. OOO designed and supervised *in silico* studies, provided resources, performed, analyzed and interpreted experiments, and wrote a section of the manuscript. OK co-supervised OAO and performed critical review. DK contributed to the experimental design, analysis, and supervision. AO performed and analyzed and interpreted experiments. IO designed, analyzed and interpreted experiments, performed literature searches, co-supervised OAO and DK, provided resources, performed critical reviews and contributed significantly to writing. All authors contributed to writing and approved the final draft.

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