FACING THE UPCOMING OF MULTIDRUG-RESISTANT AND EXTENSIVELY DRUG-RESISTANT BACTERIA: NOVEL ANTIMICROBIAL THERAPIES (NATS)

EDITED BY: Angel León-Buitimea, Jose Ruben Morones-Ramirez, Jason H. Yang and Rafael Peña-Miller

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Editorial: Facing the Upcoming of Multidrug-Resistant and Extensively Drug-Resistant Bacteria: Novel Antimicrobial Therapies (NATs)

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Editorial on the Research Topic

Facing the Upcoming of Multidrug-Resistant and Extensively Drug-Resistant Bacteria: Novel Antimicrobial Therapies (NATs)

Antimicrobial resistance is one of the largest looming threats to global health (Friedman et al., 2016), increasing the morbidity and mortality associated with bacterial infections (Ventola, 2015). ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) are responsible for the majority of nosocomial infections (Ma et al., 2020) and commonly "escape" the biocidal action of antimicrobial agents (Mulani et al., 2019). The frequent and increasing use of antibiotics in medical practice drives the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens (Magiorakos et al., 2012; Prestinaci et al., 2015). There is an urgent and critical need to design and engineer novel therapeutic alternatives for eradicating MDR and XDR bacteria through burgeoning technologies such as metal nanoparticles, genetic engineering, synthetic biology, peptide therapeutics, and combinatorial treatments. In this Research Topic, we assemble ten original articles highlighting recent discoveries around Novel Antimicrobial Therapies (NATs) against MDR and XDR bacteria.

Seven original research articles spanning diverse disciplines describe the development of NATs for clinically-relevant MDR pathogens. One study describes the one-pot synthesis of Ag-ZnO nanoparticles at low temperatures and demonstrated remarkable antimicrobial activity of these nanoparticles against methicillin-resistant *Staphylococcus aureus* (MRSA) (Naskar et al.). Another study achieved successful phytomedited synthesis of green TiO_2NPs that proved to be effective for treating biofilm-based bacterial and fungal infections (Al-Shabib et al.).

Another research article assessed the therapeutic efficacy of antimicrobial combinations on carbapenemase-producing *Enterobacterales* (CPE). The authors showed how antimicrobial combinations synergized against most CPE expressing resistance genes. These antimicrobial combinations may facilitate the successful treatment of patients infected with CPE (Zhou et al.). Another original research article identified two potent combinations of antibiotics for clinical MRSA infection, both *in vitro* and *in vivo* (Yu et al.). A separate study found that the compound

2,4-Di-Tert-Butylphenol isolated from an endophytic fungus substantially reduced the secretion of virulence factors and biofilm and its associated factors controlled by quorum sensing in a dose-dependent manner in *Pseudomonas aeruginosa* (Mishra et al.).

Furthermore, a study tested the anti-virulence activity of potential uridine diphosphate glucose pyrophosphorylase (UDPG:PP) inhibitors and showed that these inhibitors are a potential drug candidates against *Streptococcus pneumoniae* infections (Cools et al.). New putative antimicrobial candidates were reported by Okella et al. They designed an antimicrobial peptide and performed target identification based on a putative antimicrobial peptide motif derived from fish. From all the peptide motifs generated in this work, the authors identified Pleurocidin (secreted by flatfish) as having strong antimicrobial potential.

Three review articles included in this special issue address the use of NATs to face MDR bacteria. A mini-review discusses combination treatments (particularly antimicrobial peptides and metal nanoparticles) as a pathway to develop antimicrobial therapeutics with broad-spectrum antibacterial action, bactericidal instead of bacteriostatic activity, and better efficacy against MDR bacteria (León-Buitimea et al.). Another review explored the possibility of designing antimicrobial nanoparticle-based devices to exploit the potential of antimicrobial nanoparticles to combat MDR pathogens (Gómez-Núñez et al.). Finally, a third review describes the mechanisms associated with drug resistance in pyogenic streptococci and discusses the advantages and limitations of innovative therapeutic strategies such as bacteriocins, bacteriophage, phage lysins, and metal nanoparticles (Alves-Barroco et al.).

In summary, this group of articles contributes to the search for new therapeutic strategies to combat antibacterial resistance. MDR and XDR infections are growing in incidence; the main challenges facing society are now to design, develop, and evaluate new therapeutic strategies that can spearhead the development of alternative therapies against clinically-relevant MDR pathogens.

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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Easy One-Pot Low-Temperature Synthesized Ag-ZnO Nanoparticles and Their Activity Against Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*

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Antimicrobial resistance (AMR) is widely acknowledged as a global health problem, yet the available solutions to this problem are limited. Nanomaterials can be used as potential nanoweapons to fight against this problem. In this study, we report an easy one-pot low-temperature synthesis of Ag-ZnO nanoparticles (AZO NPs) and their targeted antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The physical properties of the samples were characterized by X-ray diffractometry (XRD), transmission electron microscopy (TEM), and X-ray photoelectron spectroscopy (XPS). Furthermore, minimum inhibitory concentration (MIC), zone of inhibition (ZOI), and scanning electron microscopy (SEM) images for morphological characterization of bacteria were assessed to evaluate the antibacterial activity of AZO NPs against both Gram-negative [*Escherichia coli* (*E. coli*) and *Acinetobacter baumannii* (*A. baumannii*) standard and AMR strains] and Gram-positive (*S. aureus*, MRSA3, and MRSA6) bacteria. The AZO NPs showed comparatively better antibacterial activity against *S. aureus* and MRSA strains than Gram-negative bacterial strains. This cost-effective and simple synthesis strategy can be used for the development of other metal oxide nanoparticles, and the synthesized nanomaterials can be potentially used to fight against MRSA.

Keywords: low-temperature solution synthesis, Ag-ZnO nanoparticles, antibacterial activity, Gram-positive bacteria, MRSA

INTRODUCTION

Antimicrobial resistance (AMR) is the ability of a given microbe to resist the effects of multiple antibiotics (Huijbers et al., 2015; Prestinaci et al., 2015). AMR is easily recognized in hard-to-treat pathogens and has become an alarming issue complicating health care and many other sectors (Eliopoulos et al., 2003; Jasovsky et al., 2016). For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most well-known AMR bacterial species for which immediate intervention is necessary, but even the long considered last-resort antibiotic vancomycin cannot be used in the treatment of MRSA infections since vancomycin-resistant *S. aureus* (VRSA) strains

have emerged (Naskar and Kim, 2019; Naskar et al., 2020). In addition, AMR *S. aureus* species are one of 12 families of priority pathogenic bacteria listed by the World Health Organization (WHO) for which antibiotics are urgently needed (World Health Organization [WHO], 2017). Several new currently approved oxazolidinone class antibiotics, including Sivextro (Hall et al., 2018), tigecycline (Hall et al., 2018), and LCB01-0371 (Jeong et al., 2010) to eradicate *S. aureus* species resistant to last-resort antibiotics have been developed. However, it is possible that bacteria might continue to evolve to evade this new class of last-resort antibiotics, and it takes much time to find other alternatives and their mechanism of action in response to a newly generated resistant strain. Therefore, new alternatives to antibiotics are desperately needed for the fight against AMR pathogens.

In this present scenario, nanomaterials have emerged as both viable and versatile alternatives to current antibiotics to fight against AMR bacteria as it showed effectiveness in low dosages also where chances of bacteria getting resistance is also less (Regí et al., 2019). The main advantage of nanoparticles as antibacterial agents (i.e., nanoweapons) is that they function via a multiple target approach compared to the single target approach of antibiotics to inhibit the growth of bacteria (Naskar et al., 2016; Baptista et al., 2018). Therefore, it is harder for bacteria to attain resistance toward nanoparticles. A large surface area to volume ratio is also one of the major advantages of nanoparticles for their use in various biomedical applications including antibacterial activity (Navya and Daima, 2016; Naskar et al., 2018). Among such nanomaterials, metal- and metal oxide-based nanoparticles have been preferred by researchers to combat AMR bacterial cells (Wang et al., 2017). However, silver (Ag) nanoparticles (NPs) have been the most effective and promising antibacterial candidates since ancient times due to their inhibitory and antibacterial properties against microorganisms, including 16 major species of bacteria (Lee and Jun, 2019). Moreover, zinc oxide (ZnO) NPs are another well-known antibacterial nanomaterial (Sirelkhatim et al., 2015; Hassan et al., 2017; Kumar et al., 2017; Naskar et al., 2017). ZnO nanoparticles have been recognized as a safe material by the US Food and Drug Administration [(21CFR182.8991) (Food and Drug Administration (FDA), 2015)]. Therefore, Ag-ZnO (AZO) NPs can be a potential alternative to conventional antibiotics in the fight against AMR bacteria.

Several methods like sol–gel (Lu et al., 2011) hydrothermal (Zhang and Mu, 2007) co-precipitation (Md Subhan et al., 2014), and plasma-assisted chemical vapor deposition (Simon et al., 2011) have been successfully reported for the synthesis of AZO NPs. However, all of these processes use high temperature and high pressure with long reaction times and multiple steps, which limit the use of AZO NPs in various applications (Matai et al., 2014). Very few reports, in fact, are available regarding the single step and low temperature synthesis of AZO NPs for the killing of AMR pathogens (especially MRSA pathogens) despite the immense potential for AZO NPs as antibacterial agents.

In the present work, a simple one-pot low-temperature synthesis method was developed to successfully synthesize AZO NPs from simple metal precursors and hydrazine hydrate.

The antibacterial activity of the synthesized nanoparticles was evaluated for AMR strains of Gram-positive bacteria, including MRSA strains, and Gram-negative bacteria.

MATERIALS AND METHODS

Synthesis of ZnO (ZO) and Ag-ZnO (AZO) NPs

Initially, a fixed quantity (1 g) of zinc nitrate hexahydrate ($Zn[NO_3]_2 \cdot 6H_2O$, Merck) and requisite amount of silver nitrate ($AgNO_3$, ACS, $\geq$99.9%) [0 and 5 atomic percent (at%) with respect to Zn] was uniformly dispersed in 50 mL of deionized water (DW) with continuous stirring for 60 min at room temperature. In the next step, 1 mL of hydrazine hydrate ($N_2H_4 \cdot H_2O$, Merck, 99–100%) was added dropwise to the reaction mixture with continuous stirring. Subsequently, the mixture was ultrasonicated for 10 min in a water bath ultrasonicator. Now, gray colored precipitation was clearly visible in the reaction beaker. The same steps, i.e., dropwise addition of hydrazine hydrate and ultrasonication, were repeated until the pH of the medium reached eight. Afterward, the precipitate of solid materials was separated by centrifugation and DW and ethanol were used for washing. Finally, the samples were dried in an oven at ∼60°C for 24 h. The products were designated as ZO and AZO where the at% used in the precursors was 0 and 5, respectively.

Characterization
Material Properties
X-ray diffraction (XRD) using an X-ray diffractometer (D8 Advance with DAVINCI design XRD unit, Bruker) with nickel filtered Cu Kα radiation source (λ = 1.5406 Å) was used to evaluate the structures of ZO and AZO. The diffraction patterns were collected in the 2θ range of 20–80°. Moreover, the microstructure of the representative sample of AZO was assessed by transmission electron microscopy (TEM; Bruker Nano GmbH). Carbon coated 300 mesh Cu grids were used for placing the samples. An Axis Supra Scanning X-ray photoelectron spectroscopy (XPS) microprobe surface analysis system was used to assess a representative sample of AZO by scanning the binding energy ranging from 200 to 1,200 eV to determine the chemical state of elements. The C 1s peak position at 284.5 eV was used as the binding energy reference.

Growth of Bacteria for Evaluation of the Antibacterial Activity
Generally, antibacterial activity was evaluated according to a previous report (Naskar et al., 2020) using BBL™ Mueller-Hinton Broth (MHB, Becton Dickinson) grown bacterial strains including *E. coli* (ATCC 25922), *A. baumannii* (ATCC 19606), *S. aureus* (ATCC 25923); AMR strains of *E. coli* (1368), *A. baumannii* (12001); and different MRSA clinical isolates (Shin et al., 2019). Briefly, the MHB medium was used for the inoculation of single colonies of bacteria, which were incubated at 37°C overnight, followed by dilution of the cells to an optical density of 0.5 McFarland turbidity standard

using Sensititre[TM] Nephelometer (Thermo Scientific). The cell cultures were used within 30 min after dilution to prepare samples for minimum inhibitory concentration (MIC) assay (section "MIC for Evaluation of the Antibacterial Activity") or scanning electron microscopy (SEM) analysis (section "Morphological Characterization of Bacteria") to assess the antibacterial activity of NPs (ZO and AZO) and characterize cell morphology, respectively.

MIC for Evaluation of the Antibacterial Activity

All bacteria were incubated overnight in the MHB medium. The number of cells was determined with a Sensititre[TM] Nephelometer to a 0.5 McFarland standard and diluted at a ratio of 1/1,000 in MHB. The ZO and AZO samples (5 mg/mL each) were prepared by serial dilution with DW to obtain concentrations from 250 to 10 μg/mL. Then, 90 μL of the targeted bacterial medium was inoculated with 10 μL of each diluted sample. The bacterial cells were incubated by shaking at 500 rpm for 16 h at 37°C. The MIC was evaluated after this process.

Agar Well Diffusion Method for Evaluation of the Antibacterial Activity

The antibacterial activity of ZO and AZO against the bacterial strains of *E. coli*, *A. baumannii*, *S. aureus*, MRSA3, and MRSA6 was further evaluated with the agar well diffusion method. First, 500 μL of cultured bacterial cells were mixed with 25 mL of MHB-agar, poured into sterile petri dishes (φ = 90 mm), and solidified. Then, five holes, 6 mm in diameter each, were aseptically punched through the surface with a sterile plastic rod. Afterward, 20 μL of ZO or AZO (5 mg/mL), polymyxin B or kanamycin (5 mg/mL, Sigma-Aldrich), or DW was added for the experimental group, the positive control for Gram-negative or -positive strains, and the negative control group respectively. The plates were then incubated for 24 h at 37°C. Finally, the antibacterial activities were evaluated by measuring the diameter of the zone of inhibition (ZOI) around the wells using a ruler.

Morphological Characterization of Bacteria

At first, prepared bacterial cells through the same process as described in section "Growth of Bacteria for Evaluation of the Antibacterial Activity" were diluted at a ratio of 1/1,000 in the MHB medium according to MIC assay. 900 μL of prepared cells were incubated with 100 μL of the three final concentration 0, 10, and 20 μg/mL of AZO for 16 h at 37°C with vigorous shaking. After that, the incubated cells were harvested by centrifugation at 12,000 rpm for 1 min to get a pellet. Then this pellet was resuspended in 500 μL of phosphate buffered solution (pH 7) containing 2% formaldehyde and 1% glutaraldehyde, and centrifuged again. Subsequently, the obtained cell pellet was washed twice with DW and resuspended in 1 mL of DW for further experimentation. A 5 μL aliquot was taken from the suspension and deposited on a silicon wafer (5 mm × 5 mm in size, Namkang Hi-Tech Co., Ltd.) to dry at room temperature. Finally, the air-dried wafer was subjected to SEM analysis using VEGA3 (TESCAN), a versatile tungsten thermionic emission SEM system, according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Material Properties
Phase Structure

XRD was used to analyze the crystalline phase of samples. **Figure 1** shows the XRD patterns of as-synthesized ZO and AZO samples. The obtained XRD patterns of the samples were consistent with hexagonal ZnO (h-ZnO) (JCPDS 36-1451) (Saloga and Thünemann, 2019). Moreover, some additional peaks can be seen at ∼38.1°, ∼44.3°, ∼64.5°, and ∼77.4° for AZO samples, which corresponded to the crystal planes of cubic Ag (JCPDS 04–0783) along (111), (200), (220), and (311), respectively (Nogueira et al., 2014). Therefore, the formation of AZO NPs was successfully confirmed.

Morphology and Microstructure

Transmission electron microscopy (TEM) was conducted systematically to further investigate the formation of ZO/AZO nanoparticles. The TEM image of the AZO sample and the corresponding HRTEM and HAADF images are shown in **Figures 2a–d**, respectively. The HRTEM image (**Figure 2c**) of the AZO sample shows distinct lattice fringes with an interplanar distance of 0.28 nm, corresponding to the (100) plane of hexagonal ZnO (Ren et al., 2016). This observation confirmed the presence of hexagonal ZnO in the AZO sample. Moreover, HRTEM showed lattice fringes having an interplanar distance of 0.23 nm (**Figure 2c**), which can be matched with (111) of Ag NP (Sareen et al., 2015). Therefore, the TEM characterization of the microstructure of the AZO sample confirmed the presence of both nanoparticles of ZnO and Ag, which corroborated with the XRD result (**Figure 1**). Additionally, the TEM with energy-dispersive X-ray (TEM-EDX) spectral analysis of the AZO sample confirms the presence of Zn and O and corroborates that ZnO NPs were formed (**Figure 2b**). The presence of Ag suggests the
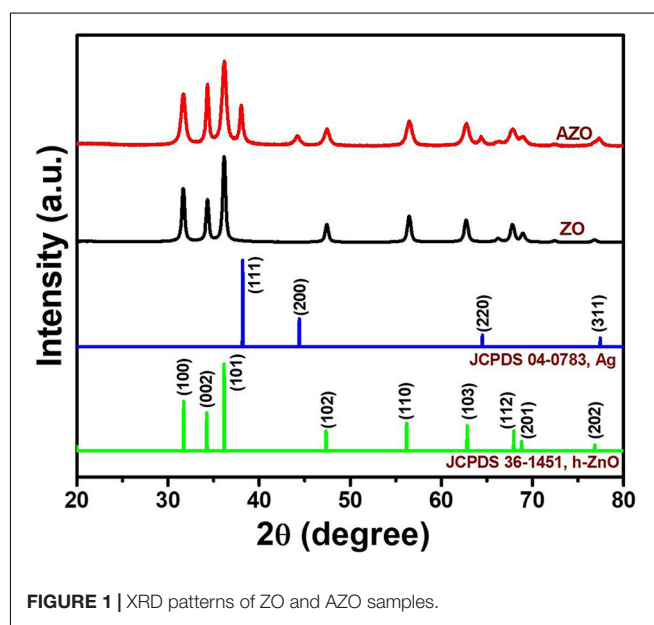
FIGURE 1 | XRD patterns of ZO and AZO samples.
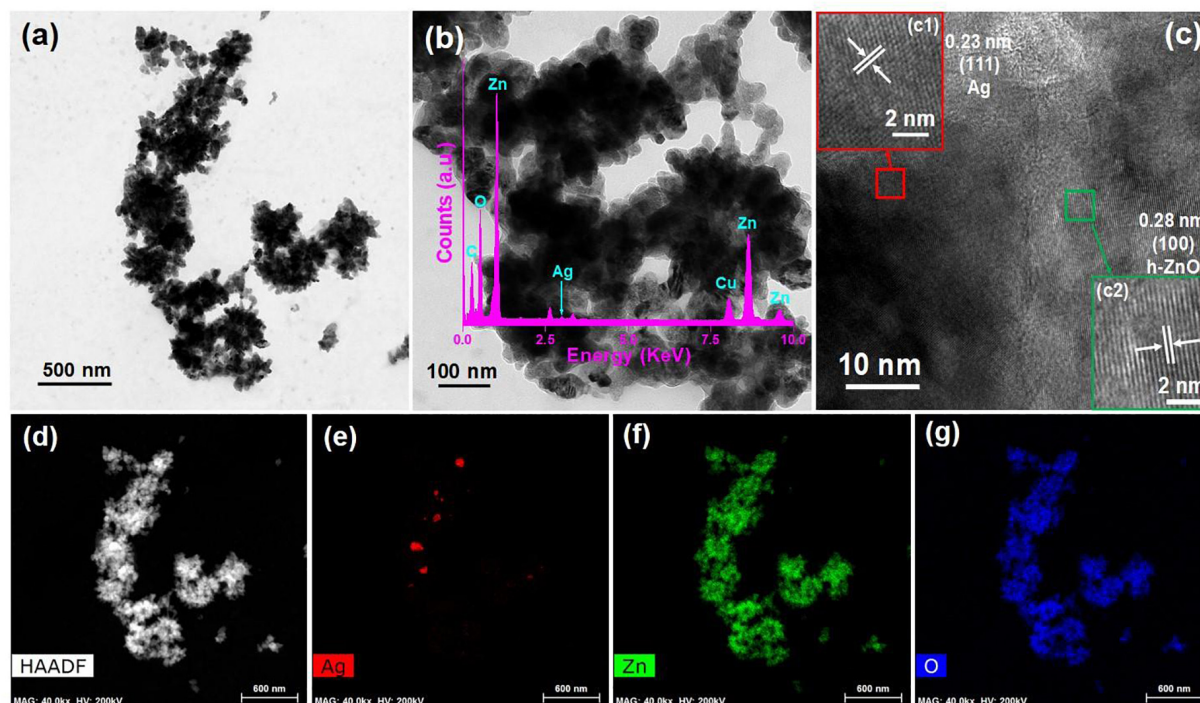
FIGURE 2 | TEM image **(a,b)** and HRTEM image **(c)** of AZO sample where **(c1)** and **(c2)** show the HRTEM images of the particles of Ag and ZnO, respectively with **(b** inset) TEM-EDS spectrum, **(d)** HAADF image, and elemental mappings of **(e)** Ag, **(f)** Zn, and **(g)** O.
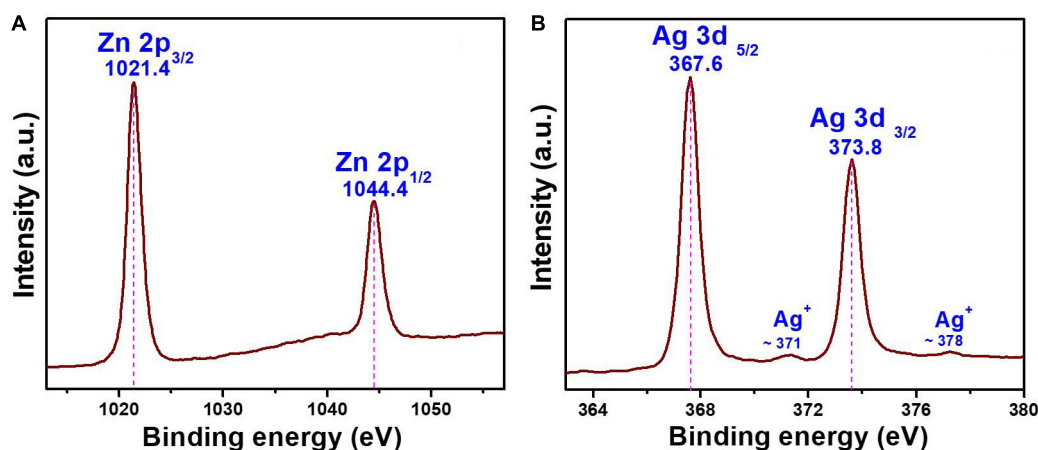
FIGURE 3 | XPS binding energy spectra of AZO **(A)** Zn *2p* and **(B)** Ag *3d* core levels.

formation of Ag NP in the Azo sample. The presence of C and Cu in the TEM-EDX spectrum can be attributed to the carbon coated Cu grid used for the TEM measurements. The elemental mapping result of Ag (**Figure 2e**), Zn (**Figure 2f**) and O (**Figure 2g**) for the representative AZO sample reveals the distribution Ag, Zn, and Au elements in the sample.

XPS Spectra

The oxidation state of the chemical elements present in the AZO sample was evaluated by XPS analysis, and the binding

energy signals of the Zn *2p* and Ag *3d* core levels are shown in **Figure 3**. Two strong signals were observed in the binding energy signals of Zn *2p* at 1021.4 and 1044.4 eV (**Figure 3A**), which can be assigned to the binding energies of Zn $2p_{3/2}$ and Zn $2p_{1/2}$, respectively (Jiamprasertboon et al., 2019). The presence of zinc as Zn^{2+} in the nanomaterial was also confirmed by the energy difference calculated between Zn $2p_{3/2}$ and Zn $2p_{1/2}$ binding energy levels, which was ∼23.0 eV (Jiamprasertboon et al., 2019). Furthermore, the formation of Ag nanoparticles was also evaluated by the binding energy signals

Bacteria cells	Minimum inhibitory concentration (μg/mL)	
	(i) ZO	**(ii) AZO**
Standard strains		
(a) *E. coli* ATCC 25922	250	100
(b) *A. baumannii* ATCC 19606	>250	250
(c) *S. aureus* ATCC 25923	50	25
AMR strains		
(d) *E. coli* 1368	>250	250
(e) *A. baumannii* 12001	>250	250
(f) MRSA3	100	50
(g) MRSA6	100	50

Minimum inhibitory concentration (MIC) of ZO and AZO samples against both Gram-negative and -positive bacteria cells including their AMR strains. Data shown here is one of the representative from n = 3.

of Ag *3d* (**Figure 3B**). The binding energy signals (**Figure 3B**) appearing at 367.6 and 373.8 eV in the XPS curve of the AZO sample can be assigned to Ag $3d_{5/2}$ and Ag $3d_{3/2}$, respectively (Nguyen et al., 2018). This observation confirmed the formation of Ag NPs in the AZO sample. Moreover, two low intensity signals can also be seen at ∼371 and ∼378 eV. These low intensity peaks can be attributed to a trace amount of Ag^+ ions present in the sample (Naskar et al., 2016). Therefore, the presence of metallic silver and vey less Ag^+

could be effectively used against bacterial cells for antibacterial activity. This material property is successfully correlated with the antibacterial activity of this sample in later section of antibacterial activity.

Antibacterial Activity
MIC and ZOI
The MIC values (**Table 1**) of the ZO and AZO samples were measured to evaluate the antibacterial effectiveness of the samples against standard strains of bacteria (*E. coli* [ATCC25922], *A. baumannii* [ATCC19606], and *S. aureus* [ATCC25923]) and AMR strains (*E. coli* 1368, *A. baumannii* 12001, MRSA3, and MRSA6). MIC determination clearly showed that the AZO sample was comparatively more effective against Gram-positive bacteria than Gram-negative bacteria. Although the AZO sample was effective against Gram-negative bacteria, its MIC was considerably very high against generic and AMR strains (100–250 μg/mL). However, the AZO sample was much more effective against Gram-positive bacterial cells; the MIC value for *S. aureus* and its AMR strains MRSA3 and MRSA6 were in the range of 25–50 μg/mL.

In additional to the MIC determination, the agar well diffusion method was also used to further evaluate the antibacterial activity of AZO NPs. Initially, agar plates with bacterial cells were loaded with the synthesized NPs (20 μL at 5 mg/mL) and incubated for 24 h at 37°C. After that, the ZOI was measured. The bacterial growth inhibition capacity of the ZO and AZO samples
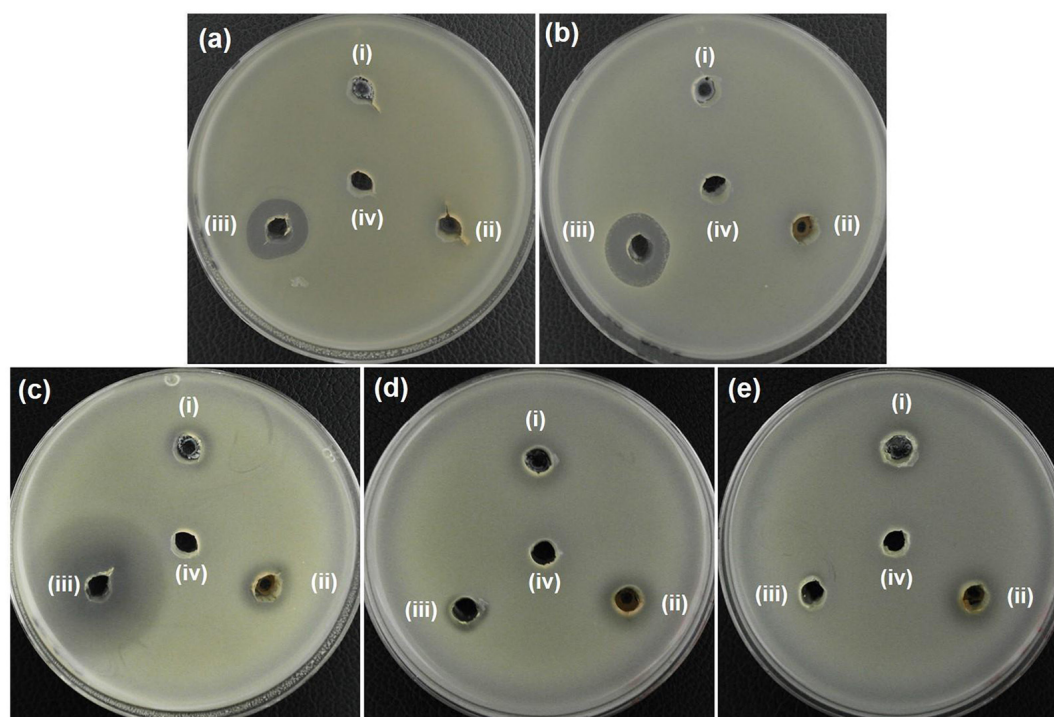
FIGURE 4 | Zone of inhibition (ZOI) of ZnO samples against **(a)** *E. coli*, **(b)** *A. baumannii*, **(c)** *S. aureus*, **(d)** MRSA3, and **(e)** MRSA6. **(i)**, **(ii)**, **(iii)**, and **(iv)** represents ZO, AZO, Antibiotics, and deionized water, respectively in all the figures. Diameter of ZOI is also displayed in the **Table 2** (average from *n* = 3).

TABLE 2 | Zone of inhibition (ZOI) diameter of ZnO samples against (a) *E. coli*, (b) *A. baumannii*, (c) *S. aureus*, (d) MRSA3, and (e) MRSA6 was measured from *n* = 3 and one of the representative data was shown.

Bacteria cells	Zone of inhibition (mm)			
	(i) ZO	(ii) AZO	(iii) Antibiotics	(iv) DW
(a) *E. coli* ATCC 25922	N.D.	N.D.	17[a]	N.D.
(b) *A. baumannii* ATCC 19606	N.D.	N.D.	19[a]	N.D.
(c) *S. aureus* ATCC 25923	11	14	37[b]	N.D.
(d) MRSA3	11	14	12[b]	N.D.
(e) MRSA6	11	13	N.D.	N.D.

N.D. indicates that the zone of inhibition was not detected. Antibiotics, [a]Polymyxin B or [b]Kanamycin was used as a control for Gram-negative or -positive bacteria, respectively. DW, deionized water. Data shown here is one of the representative from n = 3.

against *E. coli* (**Figure 4a**), *A. baumannii* (**Figure 4b**), *S. aureus* (**Figure 4c**), MRSA3 (**Figure 4d**), and MRSA6 (**Figure 4e**) is provided in **Table 2**. The ZO and AZO NPs were unable to form an inhibition zone against Gram-negative bacterial cells of *E. coli* (**Figure 4a**) and *A. baumannii* (**Figure 4b**). These results support the MIC data (**Table 1**) for Gram-negative bacterial cells, from which it can be concluded that a more concentrated dispersion of AZO NPs would be necessary to obtain an inhibition zone i.e., to be effective against Gram-negative bacterial cells in the agar well diffusion antimicrobial determination. The ZOI against AMR strains (*E. coli* 1368, *A. baumannii* 12001) of Gram-negative bacterial cells was not determined, as it was assumed to be higher than the AZO sample, which was above the limit of detection for the concentration of AZO NPs used.

However, the AZO sample was effective in inhibiting the growth of Gram-positive bacterial cells including *S. aureus* (**Figure 4c**), MRSA3 (**Figure 4d**) and MRSA6 (**Figure 4e**). This observation corroborated the MIC determinations (**Table 1**). The effectiveness of the synthesized sample of AZO against the MRSA strains substantiates its potential to be used as a nanoweapon against AMR Gram-positive bacterial cells. Additionally, the MIC and ZOI data indicate that Gram-positive bacteria are better targets for AZO NPs than Gram-negative bacteria.

Morphological Characterization of Bacteria

Given the antimicrobial efficacy of AZO NPs against Gram-positive bacteria, the morphological features of standard and AMR strains of *S. aureus* (standard, MRSA3, and MRSA6) before and after exposure to AZO nanoparticles were evaluated by SEM. The SEM images of bacterial cells treated or untreated with AZO NPS is shown in **Figure 5**. In the untreated *S. aureus* cells, a smooth and intact surface was clearly visible (**Figure 5a**). On the other hand, some morphological changes such as membrane damage were seen in *S. aureus* treated with different concentrations of AZO (**Figures 5b,c**). Similar activity was seen in MRSA strains (MRSA3 and MRSA6) when comparing the untreated groups (**Figures 5d,g**), which both exhibited smooth surfaces, with the groups treated with different
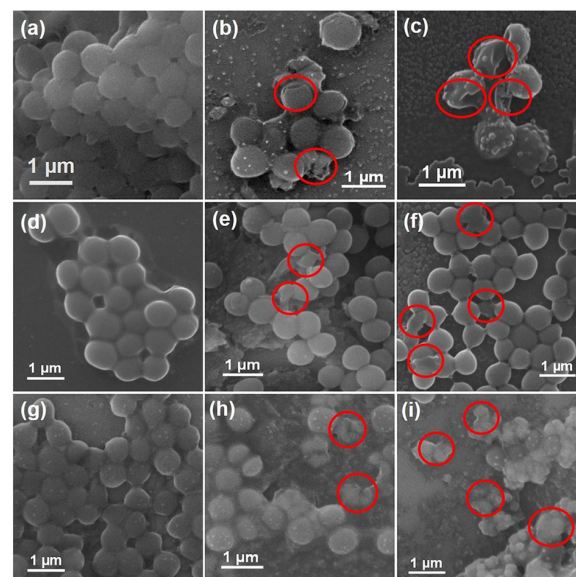
FIGURE 5 | Scanning electron microscopy (SEM) images of bacterial cells. Samples of *S. aureus* **(a)** untreated and treated with **(b)** 10 μg/mL and **(c)** 20 μg/mL of AZO. Samples of MRSA3 either **(d)** untreated or treated with **(e)** 10 μg/mL and **(f)** 20 μg/mL of AZO. Samples of MRSA6 either **(g)** untreated or treated with **(h)** 10 μg/mL and **(i)** 20 μg/mL of AZO. Red circles indicate areas of cell membrane disruption.

concentration of AZO NPs for MRSA3 (**Figures 5e,f**), and MRSA 6 (**Figures 5h,i**) which showed wrinkling and damage of the cell walls. Considerable damage was observed upon binding of the nanoparticle to the bacterial cell membrane (**Figures 5e,f,h,i**) to confirm the antibacterial effectiveness of AZO NPs. Therefore, the efficacy of AZO NPs against *S. aureus* and MRSA strains was successfully approved by the SEM micrographs.

It is well known that Ag and ZnO NPs are established antibacterial agents; however, very little is known about their mechanism of antibacterial activity. In this study, we explored one possible mechanism of Ag and ZnO NP antibacterial activity. It has been shown that some of the antibacterial activity of Ag and ZnO NPs may be attributed to a direct interaction between AZO NPs and the bacterial cell wall (Matai et al., 2014). The bacterial cell wall is generally negatively charged (Ghosh et al., 2012), which enables electrostatic interaction with the Zn^{2+} and Ag^+ present in AZO NPs (which were identified in our ZO and AZO NPs by TEM analysis and XPS). Disruption of the bacterial cell membrane by Ag-ZnO NPs can be another potential mechanism for antibacterial activity (Naskar et al., 2020), which we have corroborated here using SEM. Membrane damage generally results in increased inhibition of DNA/plasmid replication by Zn^{2+}/Ag^+ ions and the production of proteins/enzymes that affect bacterial cell functioning and contribute to cell death. Moreover, membrane disruption can also cause leakage of the intracellular material, which may shrink the cell and ultimately result in cell lysis (Yasir et al., 2019).

CONCLUSION

In summary, we have developed a new strategy for the one-pot synthesis of Ag-ZnO (AZO) nanoparticles using a low-temperature solution technique. The synthesized AZO sample showed admirable antibacterial activity against *S. aureus* bacteria including their AMR (MRSA) strains. Moreover, the antibacterial activity of the AZO sample was more specific toward Gram-positive bacteria than Gram-negative bacteria. This cost-effective simple synthesis strategy can be used as a platform to develop different metal oxide nanomaterials, which can be further used for targeted biomedical applications and may be useful as antibacterial agents to address the ever-increasing problem of AMR bacteria.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

AN, SL, and KK contributed conception and design of the study, and wrote the manuscript. AN and SL performed the experiments. KK supervised the study. All authors contributed to the manuscript revision, and read and approved the submitted version.

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In vitro and *in vivo* Evaluation of *in silico* Predicted Pneumococcal UDPG:PP Inhibitors

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Pneumonia, of which *Streptococcus pneumoniae* is the most common causative agent, is considered one of the three top leading causes of death worldwide. As seen in other bacterial species, antimicrobial resistance is on the rise for this pathogen. Therefore, there is a pressing need for novel antimicrobial strategies to combat these infections. Recently, uridine diphosphate glucose pyrophosphorylase (UDPG:PP) has been put forward as a potential drug target worth investigating. Moreover, earlier research demonstrated that streptococci lacking a functional *galU* gene (encoding for UDPG:PP) were characterized by significantly reduced *in vitro* and *in vivo* virulence. Therefore, in this study we evaluated the anti-virulence activity of potential UDPG:PP inhibitors. They were selected *in silico* using a tailor-made streptococcal homology model, based on earlier listerial research. While the compounds didn't affect bacterial growth, nor affected *in vitro* adhesion to and phagocytosis in macrophages, the amount of polysaccharide capsule was significantly reduced after co-incubation with these inhibitors. Moreover, co-incubation proved to have a positive effect on survival in an *in vivo Galleria mellonella* larval infection model. Therefore, rather than targeting bacterial survival directly, these compounds proved to have an effect on streptococcal virulence by lowering the amount of polysaccharide and thereby probably boosting recognition of this pathogen by the innate immune system. While the compounds need adaptation to broaden their activity to more streptococcal strains rather than being strain-specific, this study consolidates UDPG:PP as a potential novel drug target.

Keywords: *Streptococcus pneumoniae*, GalU, *in silico* modeling, virulence, *Galleria mellonella*, novel drug target

INTRODUCTION

Streptococcus pneumoniae is one of the major causative agents of community acquired pneumonia and meningitis worldwide. Pneumonia is one the major causes of mortality in children under the age of five and is considered to be the third leading cause of death worldwide (Marangu and Zar, 2019; Peyrani et al., 2019). In 2015, it has been reported that 64% of child deaths due to pneumonia were caused by bacterial agents *S. pneumoniae* or *Haemophilus influenzae* (Marangu and Zar, 2019). Antibacterial treatment often consists of macrolides, amoxicillin, fluoroquinolones or cephalosporins (Peyrani et al., 2019). However, an increase in resistance toward macrolides has

been reported (Yayan, 2014). For bacterial meningitis, *S. pneumoniae* is the causative pathogen in no less than 70% of all cases. While vaccination proved successful, there is now a re-emergence of pneumococcal infections due to serotype replacement, leading to incidences equal to the pre-vaccination era in some parts of Europe and North America (Koelman et al., 2019).

Amongst the variety of virulence factors the pneumococcus possesses, the polysaccharide capsule is considered as the most important one (Paton and Trappetti, 2019). Due to its presence, the first line of defense to pneumococcal invasion, i.e., macrophage phagocytosis, is limited and there is no adequate T-cell response (Geno et al., 2015). Deletion of this capsule drastically reduces pneumococcal virulence by increasing phagocytosis rates (MacLeod and Kraus, 1950; Preston and Dockrell, 2008). *In vitro*, non-encapsulated pneumococci show better adherence properties and also in *in vivo* nasopharyngeal colonization a decrease in capsule production is observed (Kadioglu et al., 2008; Gilley and Orihuela, 2014). However, when the transition from a commensal to an invasive lifestyle occurs, there is a clear upregulation of capsule production, likely due to the importance of it in evading the immune system (Kadioglu et al., 2008).

The most important gene locus for capsule production is the gene locus, which gives rise to over 90 different pneumococcal serotypes. Interestingly, these serotypes differ in the composition of the polysaccharide capsule, with a variety of sugars that can be included. A common feature in all serotypes is the presence of glucose (Glc) and/or galactose (Gal) (Geno et al., 2015; Paton and Trappetti, 2019). Apart from the *cps* gene locus, other genes are also known to be involved in the regulation of capsule production (Llull et al., 1999). One of these genes is the highly conserved *galU* gene, which encodes for uridine diphosphate glucose pyrophosphorylase (UDPG:PP, EC2.7.7.9). Briefly, UDPG:PP reversibly converts uridine diphosphate glucose (UDP-Glc) to glucose-1-phoshate (Glc-1-P) as part of the Glc and Gal metabolism. Furthermore, UDP-Glc is a key component in the formation of pneumococcal capsule (Mollerach et al., 1998). It has been shown that mutants lacking a functional *galU* gene do not form any detectable amount or at least show a significant downregulation of capsular polysaccharide (Mollerach et al., 1998; Cools et al., 2018). Moreover, *galU* mutants are more prone to *in vitro* macrophage phagocytosis and considerably less virulent *in vivo* (Cools et al., 2018). In addition, while UDPG:PP is present in almost all life on earth, prokaryotic UDPG:PPs are structurally unrelated to their eukaryotic counterparts (Flores-Díaz et al., 1997; Berbís et al., 2015). Also in other organisms, UDPG:PP alteration has been suggested as a way of battling infection, e.g., against *Escherichia coli, Klebsiella Pneumoniae*, and *Pseudomonas* (Berbís et al., 2015). We therefore postulate the UDPG:PP enzyme could present a potential effective drug target against *S. pneumoniae* as well.

As the crystal structure of pneumococcal UDPG:PP is currently unknown, it's exact conformation and location of the binding site is unsure. Therefore, a recently published computational model based on listerial UDPG:PP was optimized for *S. pneumoniae* (Kuenemann et al., 2018). Adaptation of this model led to the identification of several hit compounds, which were characterized, predicted and selected *in silico* using 3D molecular docking in order to have a binding affinity that could result in some enzyme inhibitory activity. Three of these compounds were then evaluated in several *in vitro* and *in vivo* assays. Our main findings are that the tested potential inhibitors were indeed capable of modulating virulence. Moreover, this effect was dependent on the bacterial strain used, potentially enabling strain- and pathogen-specific virulence modulation. Therefore, more research should be done concerning these modulators in order to fully establish their bioprofiles and allow for a broader spectrum of inhibition in pneumococci. Overall, our data further establish UDPG:PP as a potential drug target against *S. pneumoniae* infections and confirm the significance of anti-virulence therapies as a promising avenue for fighting bacteria.

MATERIALS AND METHODS

Homology Modeling and Protein Preparation

Homology models were built using Prime's energy-based method included in the Schrödinger software suite based on D39/R6 and TIGR4 strains sequences (Jacobson et al., 2002, 2004). As template our in-house *Listeria monocytogenes* UDPG:PP 3D structure was used (Kuenemann et al., 2018). D39/R6 and TIGR4 share 98% of sequence identity based on ClustalW alignment (**Supplementary Figure S1**; Goujon et al., 2010). Meanwhile, these two *S. pneumoniae* strains share 63% of sequence identity and 78% of sequence similarity with *L. monocytogenes*. Once built, the models were standardized using the Protein Preparation Wizard from the Schrödinger Suite to ensure that there was no missing/clashing atom (including hydrogens). H-bonds assignment were performed at pH = 7 with PROPKA, and an additional energy minimization was performed with the OPLS3 force field (Harder et al., 2016). Obtained homology models were aligned on the listerial in-house structure, and Cα RMSD (Root Mean Square Deviation) were calculated. RMSD between the streptococcal strain D39/R6 homology model and *L. monocytogenes* is equal to 0.17 Å, whereas RMSD between streptococcal strain TIGR4 and *L. monocytogenes* is equal to 0.18 Å. The two *S. pneumoniae* homology models are available in the **Supplementary Material** and **Supplementary Figure S2**.

Molecular Docking

Molecular docking of the 37 UDPG:PP ligands extracted from our previous study was done using both strains D39/R6 and TIGR4 homology models using Glide 2019-1 (Kuenemann et al., 2018). Extra precision (XP) mode with flexible ligand sampling was done following the same protocol as in our previous work (Friesner et al., 2004, 2006; Halgren et al., 2004; Kuenemann et al., 2018). Moreover, the same parameters were kept to generate the grid box using Receptor Grid Generation from Schrödinger. The outer box of $30 \times 30 \times 30$ Å defines the volume in which the grid potentials are computed. The grid center has as coordinates $x = 2.10$, $y = 46.44$, and $z = 14.85$. The inner box of $10 \times 10 \times 10$ Å represents the volume where the ligand center must be placed.

The docking calculations allowed us to obtain, visualize and study the potential binding modes of the 37 listerial UDPG:PP inhibitors in the binding pockets for *S. pneumoniae* strains.

Bacterial Strains, Cell Cultures and Compounds

All *S. pneumoniae* strains used in this study are listed in **Table 1**. Strains 85, 85 *galU* mutant, M23 and M23 *galU* mutant were a kind gift from Prof. Mollerach, Universidad de Buenos Aires, Argentina. Briefly, *galU* mutants were obtained through an interruption in the last 102 nucleotides of the gene leading to deletion of the last 33 C-terminal amino acids of the enzyme, which in turn leads to a disorganization of the enzyme tetramer (Mollerach et al., 1998; Martin et al., 2000; Cools et al., 2018). Reference strain TIGR4 (serotype 4) was obtained from ATCC® (ATCC® BAA-334™). Reference strains D39 (serotype 2) and R6 (serotype 2⁻, unencapsulated) were obtained from NCTC® (NCTC07466 and NCTC13276, respectively). Bacteria were cultured in brain-heart infusion (BHI) broth (LabM) or on 5% sheep blood agar plates (Tryptic Soy Agar, LabM, Oxoid) at 37°C and 5% CO_2. Murine macrophage cells were obtained from ATCC® (RAW 264.7, ATCC® TIB-71™) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal calf serum (IFCS) and 1% pyruvate (all from Sigma-Aldrich) under the same conditions. Three compounds were purchased from Asinex Corporation (BDH 33910157, BDG 33920985, BDH 33910188). Compound structures, properties and codes provided by the vendor are listed in **Table 2**. Compounds were suspended upon arrival in dimethyl sulfoxide (DMSO) (Novolab) to 10 mM and stored in the dark at room temperature.
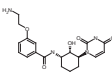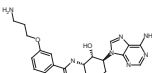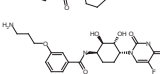
Planktonic Growth

Planktonic growth curves were obtained over an 8-h period. All strains were grown in BHI broth at 37°C and 5% CO_2, as advised by ATCC®, with or without 50 μM of compound. At

TABLE 1 | *S. pneumoniae* strains used in this study.

S. pneumoniae strain	Serotype	Source
85	Serotype 14	Prof. Mollerach, Universidad de Buenos Aires, Argentina
85 *galU* mutant	Serotype 14, *galU* mutated	Prof. Mollerach, Universidad de Buenos Aires, Argentina
M23	Serotype 3	Prof. Mollerach, Universidad de Buenos Aires, Argentina
M23 *galU* mutant	Serotype 3, *galU* mutated	Prof. Mollerach, Universidad de Buenos Aires, Argentina
TIGR4	Serotype 4	ATCC®, BAA-334
D39	Serotype 2	NCTC®, NCTC07466
R6	Derived from serotype 2, capsule deficient	NCTC®, NCTC13276

TABLE 2 | Compounds used in the *in vitro* and *in vivo* assays.

Asinex name	Structure	Molecular weight (g/mol)
BDH 33910157		388.43
BDH 33910188		425.49
BDG 33920985		436.44

All compounds were purchased from Asinex Corporation and were resuspended in DMSO to a stock concentration of 10 mM.

2-h intervals, the concentration was determined by viable plate count. For each strain and compound, 3 independent repeats were carried out.

Antibiotic Activity

Minimal inhibitory concentrations (MIC) of all compounds were determined using a resazurin assay as described previously (Torfs et al., 2018). Briefly, 100 μL of a ¹/₂ serial dilution series of compounds in BHI broth was added to 96-well plates, after which bacteria were added to a final concentration of 2.5×10^5 CU/mL in 200 μL. The highest concentration of compounds was 64 μM after addition of bacteria. After 20 h of incubation at 37°C and 5% CO_2, 20 μL of 0.005% resazurin was added. After an additional incubation of 90 min, fluorescence was measured at $\lambda_{emission} = 590$ nm, $\lambda_{excitation} = 550$ nm using a spectrophotometer (Promega Discover). For each strain, 2 independent repeats were carried out.

Cytotoxicity Assay

MRC-5 cells were grown into polystyrene 96-well plates at an initial concentration of 1.5×10^5 cells/mL cells per well and incubated at 37°C and 5% CO_2. In each well, 190 μL of cell suspension was added together with 10 μL of watery compound dilutions. Cell growth was compared to untreated control wells (100% cell growth) and medium-control wells (0% cell growth). After 3 days of incubation, cell viability was assessed using resazurin as described earlier. A compound is classified non-toxic when the IC_{50} is greater than 20 μM. Tamoxifen was used as a positive control.

Macrophage Assay

RAW 264.7 cells were seeded into polystyrene 24-well plates at 2×10^5 cells per well and incubated at 37°C and 5% CO_2, 24 h prior to infection. Bacteria were grown as described earlier for 4 h prior to infection, with or without 50 μM of compound. Then, bacteria were added to cells at a multiplicity of infection (MOI) of 10 in DMEM + 10% iFCS + 1% pyruvate as described previously (Domon et al., 2016). Plates were incubated for 90 min at 37°C and 5% CO_2. Cells were washed twice with PBS/$Ca^{2+}Mg^{2+}$, to wash away all loose bacteria. For determination of intracellular bacteria, 50 mg/mL gentamicin (Life Technologies) was added at 200 μL/mL in DMEM + 10% iFCS + 1% pyruvate. Cells were incubated for 60 min at 37°C and 5% CO_2 to kill all extracellular

bacteria. Afterward, cells were lysed using 200 μL 0.1% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature and the concentration of internal bacteria was determined using the viable plate count method. To determine the total amount of intracellular and adhered bacteria, 200 μL 0.1% Triton X-100 was added directly after washing the cells and the concentration was determined by viable plate count. For each strain, three independent repeats were carried out.

FITC-Dextran Exclusion Assay

The degree of encapsulation was determined by measuring the zone of exclusion of FITC-dextran (200 kDa, Sigma-Aldrich), as described previously (Gates et al., 2004; Weinberger et al., 2009; Cools et al., 2018). Briefly, bacteria were grown as described earlier until logarithmic phase with or without 50 μM of compound, centrifuged for 5 min at 5000 g and resuspended in PBS. To 100 μL of bacterial suspension 20 μL FITC-dextran (10 μg/mL in water) was added and 10 μL of this suspension was subsequently used to create wet mounts with cover slips. The slides were viewed on a Zeiss Observer.Z1 microscope with a 100x objective, and fluorescent images were captured with a Zeiss AxioCam MRm camera. The images were converted into grayscale and analyzed using ImageJ software. The area of FITC exclusion was determined. For each fluorescent image, a brightfield image was also recorded in order to count the number of bacteria per chain. For each strain, the mean of 150–300 cells was determined, and at least two images were collected from each of at least two independently prepared slides.

Galleria mellonella Killing Assay

Larvae were purchased from a local vendor (Anaconda Reptiles, Kontich, Belgium) and stored in wood chips at 15°C before use. Four hours before use, larvae were put at 4°C. A sterile 20 μL Hamilton syringe was used to inject 10 μL aliquots of bacterial suspensions into the hindmost left proleg of *Galleria mellonella*. Bacteria were grown mid logarithmic phase for 6 h with or without 1, 10, 50 or 200 μM compounds, washed and resuspended in PBS before infection. The control group was injected with 10 μL PBS. A minimum of 10 larvae per group was used. Following the injections, larvae were incubated at 37°C in the dark for several days to allow progression of the pneumococcal infection. Every 24 h, larvae were scored as dead or alive. Larvae were determined dead when no signs of movement could be observed in response to external stimuli. For each strain, at least four independent repeats were carried out.

Statistical Analysis

Data were analyzed for statistical significance using Graphpad Prism Version 8. Continuous variables were compared by one-way Anova, two-way Anova, t-test or survival analyses. Statistical significance was defined as $P < 0.05$. Statistical significance between groups is mentioned as asterisks in figures (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

RESULTS

Molecular docking of the 37 *listerial* UDPG:PP hit compounds from our previous study (Kuenemann et al., 2018) was performed toward the newly built strains D39/R6 and TIGR4 UDPG:PP homology models, as described in the "Materials and Methods" section. First, the reproducibility of our docking results regarding listerial UDPG:PP using the newest version of 2019 Glide was tested. While some minor differences in the docking scores (DS) obtained by the best poses were observed, overall the most potent compounds (BDG 33920985, BDF 34002917, BDH 33911533, and BDH 34012219) were still ranked in the top 5 (**Table 3**). This rarely reported reproducibility test was critical for ensuring the validity of the following docking calculations.

Regarding the docking for streptococcal UDPG:PP, most of the resulting DS were found to be in the same binding affinity range. There were a few exceptions: for instance, we noted that BDH 33910157 obtained a very good DS for listerial UDPG:PP but not at all for *S. pneumoniae* models (**Supplementary Table S1**). The compounds achieving the best DS (thus lowest values) were BDH 33911485, BDH 33910188, BDF 34002917, BDH 34000291, and BDG 33920985.

Three compounds were selected to perform the experimental study according to their DS and to their availability in Asinex stock, i.e., BDG 33920985 (active on *L. monocytogenes* and predicted to be active toward D39/R6), BDH 33910157 (only active on *L. monocytogenes*, as negative control) and BDH 33910188 (predicted to be active on D39/R6 and TIGR4). Their protein-ligand interactions were studied and the associated residues are reported in **Supplementary Figure S3**. Several residues including Pro11, Glu29, Arg108, Asp132, Ile208, and Gln234 are predicted to be critical for the binding mode of the compounds (**Figure 1**). For instance, Asp132 is predicted to establish a strong H-bond with the terminal amine moiety

TABLE 3 | Docking scores (DS) in kcal/mol for top five ranked compounds.

	Model	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5
S. pneumoniae	D39/R6	**BDH 33911485** (−8.92)	**BDH 34000291** (−8.37)	**BDH 33910188** (−8.35)	BDH 33911472 (−8.30)	BDG 33920985 (−8.27)
	TIGR4	BDF 34002917 (−9.31)	**BDH 33911485** (−9.17)	**BDH 34000291** (−9.06)	**BDH 33910188** (−8.46)	BDH 33920962 (−7.58)
L. monocytogenes	In-House redocking 2019	BDG 33920985 (−9.93)	BDF 34002917 (−9.29)	BDH 33911533 (−8.86)	BDH 34012219 (−9.03)	BDH 34012595 (−8.39)
	Kuenemann et al., 2018	BDG 33920985 (−10.02)	BDH 33910157 (−9.59)	BDF 34002917 (−9.20)	DH 33911533 (−9.16)	BDH 34012219 (−9.03)

In bold are compounds ranked as top five for both Streptococcus pneumoniae strains.
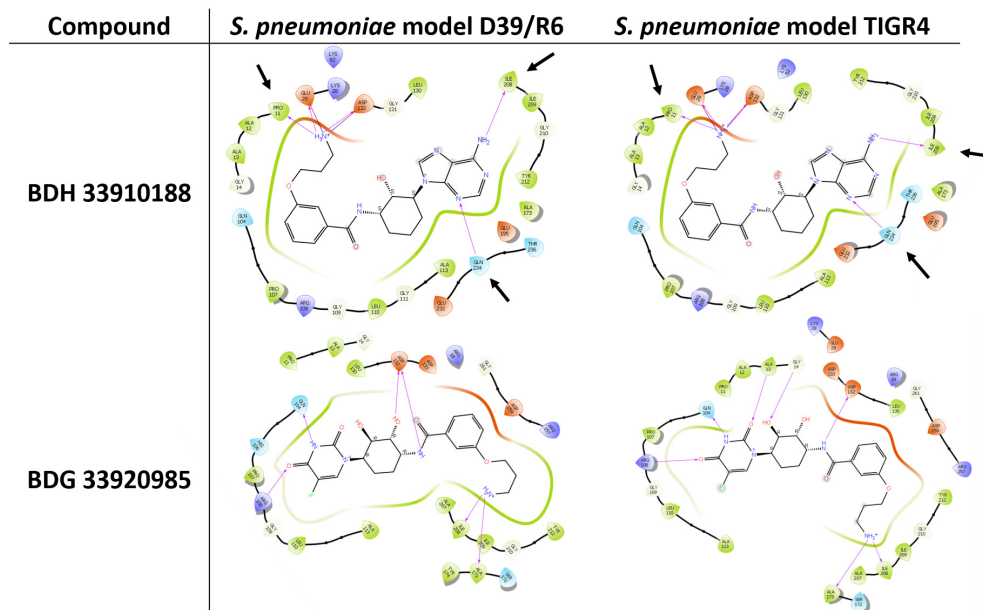
FIGURE 1 | Illustration of protein-ligand interaction. Compounds BDH 33910188 and BDG 33920985 with UDPG:PP of streptococcal strains D39/R6 and TIGR4. Arrows point toward the residues which could be responsible for the improvement in the docking score (DS) in the model. For compound BDH 33920985, DS of *S. pneumoniae* models were lower than those of the *L. monocytogenes* models.

of BDH 33910188 and the amide group of BDG 33920985. Similarly, Arg108 could be of importance for the anchoring of BDG 33920985.

In vitro Bacterial Growth Is Unaffected

Since UDPG:PP is part of the glucose and galactose metabolism, inhibition of this enzyme could potentially lead to changes in growth characteristics. To assess whether addition of the compounds to growing bacterial cultures had an effect on pneumococcal viability, planktonic growth in the presence of these compounds was evaluated. As seen in **Figure 2**, addition of the compounds didn't alter planktonic pneumococcal growth for any pneumococcal strain ($p > 0.05$, Two-way Anova). Also, antimicrobial properties of the compounds were evaluated using a standard antimicrobial susceptibility test. Again, even the highest tested concentration of compounds, 64 μM, didn't result in a decrease in viability. Taken together, these results indicate the potential inhibitors have no effect on pneumococcal viability or survival, which in light of the ongoing battle against antimicrobial resistance, is an important feature. Lastly, cytotoxicity of the compounds to MRC-5 cells was determined. Compounds BDH 33910157 and BDH 33010188 showed an CC_{50} over the maximal tested concentration of 64 μM, for compound BDG 33920985 the CC_{50} was 43.7 μM. As compounds are considered cytotoxic when the CC_{50} is below 20 μM, none of the compounds showed cytotoxic activity.

Capsule Production Is Lowered but *in vitro* Phagocytosis Remains Unaltered

To evaluate the effect of the compounds on the amount of capsule produced by *S. pneumoniae*, the bacteria were measured using the FITC-dextran exclusion assay. This assay measures the size of the bacteria, including their capsule. While a polysaccharide capsule is not visible using a regular brightfield light microscope, fluorescence microscopy can be used. As fluorescently labeled dextrans are unable to pass the polysaccharide barrier, the size of fluorescent exclusion can be directly linked to the size of the bacteria and thus to the amount of polysaccharide capsule. The compounds slightly, but significantly, lower the size of streptococcal strains TIGR4, R6, 85 and the *galU* mutant of strain M23 (**Figures 3A,C,D,G**) ($p < 0.0001$ for all combinations, except strain 85 – BDH 33910157: $p = 0.0297$, One-way Anova). Only compounds BDH 33910157 and BDH 33910188 had no effect on the size of strains R6 and M23 *galU* mutant, respectively (strain R6 – BDH 33910157, $p = 0.0709$, strain M23 *galU* mutant – BDH 33910188: $p = 0.8351$, One-way Anova). At least for strains R6 and M23 *galU* mutant, which were both already capsule deficient prior to co-incubation with compounds, this implies another mechanism of action of these compounds where e.g., also the cell wall of the bacteria or the glucose metabolism is involved (Berbís et al., 2015). However, not all capsule deficient strains show an additional decrease in size. The compounds had no effect on the size of strain 85 *galU* mutant (**Figure 3E**) (strain 85 *galU*
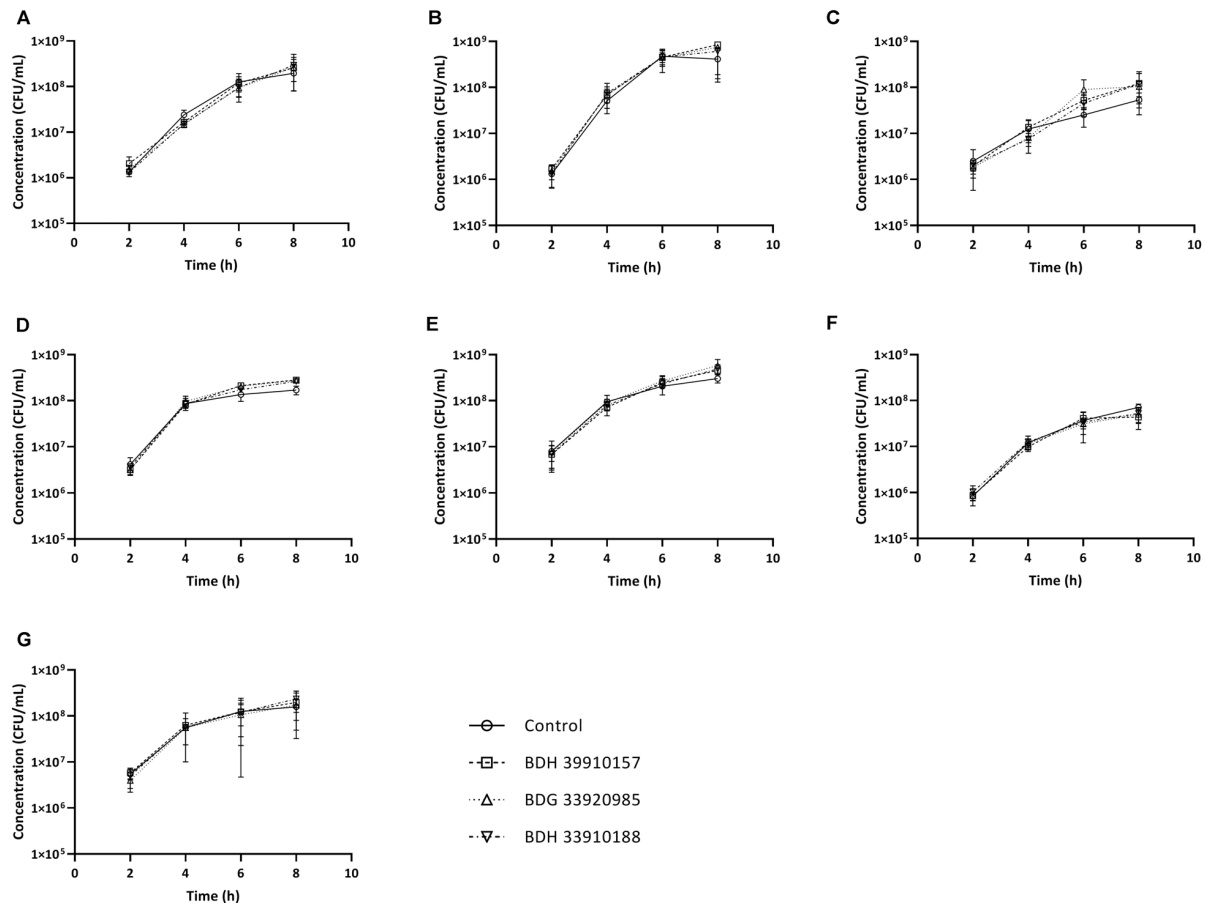
FIGURE 2 | Planktonic growth curves for *S. pneumoniae* strains in presence or absence of 50 µM of compound. **(A)** Strain TIGR4, **(B)** Strain D39, **(C)** Strain R6, **(D)** Strain 85, **(E)** Strain 85 *galU* mutant, **(F)** Strain M23, **(G)** Strain M23 *galU* mutant. Error bars represent SD. No statistical differences between control groups and treatment groups were observed (two-way Anova) (*n* = 3).

mutant – BDH 33910157: *p* = 0.3378, strain 85 *galU* mutant – BDG 33920985: *p* = 0.9701, strain 85 *galU* mutant – BDH 33910188: *p* = 0.9760, One-way Anova). Lastly, in strains D39 and M23 the compounds had an opposite effect, as co-incubation with compounds increased the area of exclusion, thus increased bacterial size (**Figures 3B,F**) (*p* < 0.0001 for all combinations, One-way Anova). In strain D39 these effects are rather limited, but in strain M23 – which is the largest of all pneumococcal strains used in this study – the compounds have a profound effect on bacterial size. Off topic, capsule production was significantly lower in *galU* mutant strains 85 and M23 compared to their respective parent strains (*p* < 0.0001 in both cases, Unpaired *t*-test). As the polysaccharide capsule is the most predominant factor in macrophage adhesion and phagocytosis, differences in bacterial size are postulated to lead to differences in cellular interactions. As reported before, *galU* mutated strains clearly show an increase in macrophage phagocytosis compared to their non-mutated parent strains (strain M23 – strain M23 *galU* mutant: *p* < 0.0001, strain 85 – strain 85 *galU* mutant: *p* = 0.0004, Unpaired *t*-test) (**Figure 4**; Cools et al., 2018). However, addition of the compounds rendered no changes in phagocytosis rates.

Even for strain M23, where the largest variation in size was recorded, no change in adherence or phagocytosis was seen. This implies that either the changes in polysaccharide production are not diverse enough to provoke changes or that the assay is not sensitive enough to pick up on them.

In vivo Virulence of Pneumococci Is Attenuated

As the macrophage assay proved no changes in macrophage functionality, a difference in *in vivo* virulence was not expected. In order to assess virulence of all virulent pneumococcal strains (TIGR4, D39, M23, and 85) and the effect of addition of compounds, a *G. mellonella* infection model was used. This model is easy to use, cheap and it is possible to set up large experiments including several variables (Cools et al., 2019). Contrary to prior expectations, there was an effect of the compounds in several virulent pneumococcal strains (**Figure 5**). Co-incubation with compounds lead to a decrease in virulence for strains TIGR4 and 85 (**Figures 5A,C**) (strain TIGR4 – BDH 33910157: *p* = 0.0005, strain TIGR4 – BDG 33920985: *p* = 0.0193, strain TIGR4 – BDH 33910188: *p* = 0.0016, strain 85 – BDH
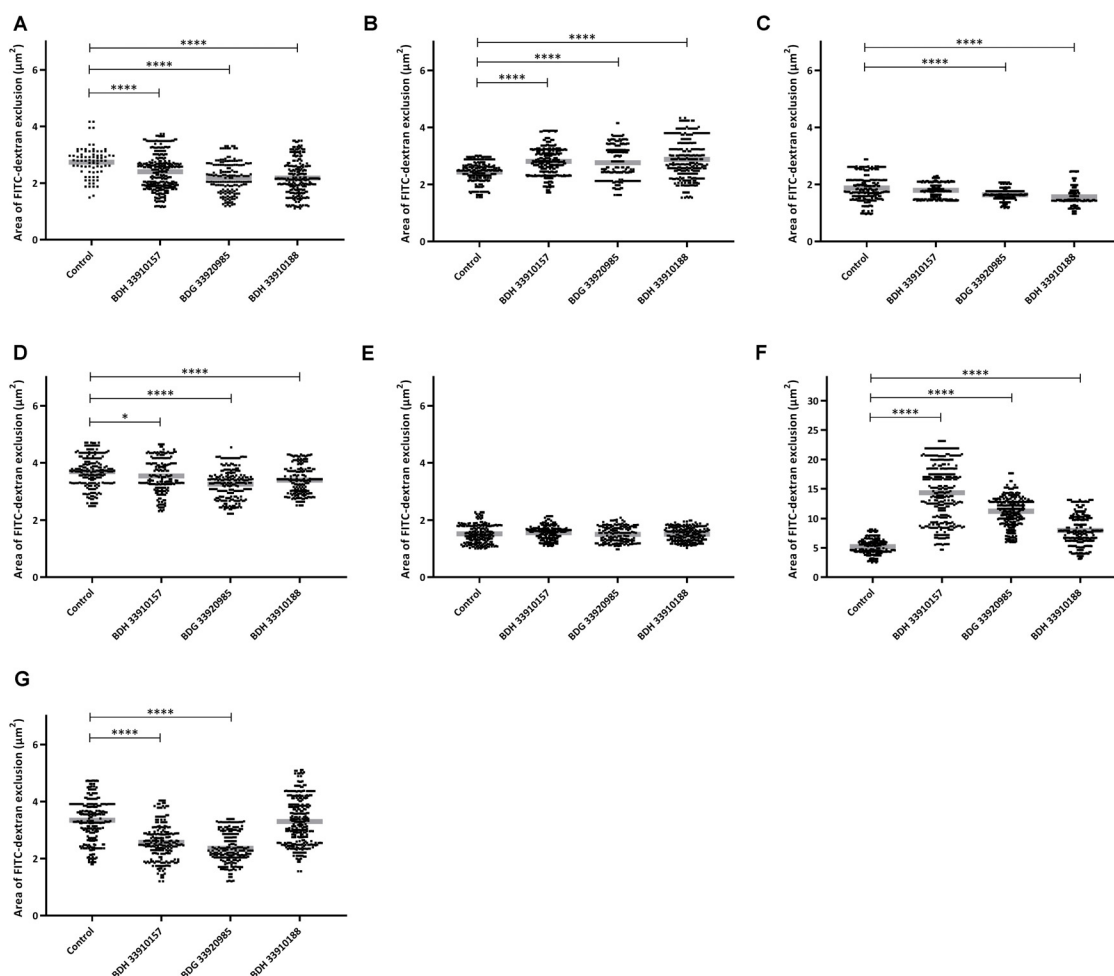
FIGURE 3 | FITC-dextran exclusion assay. Area of exclusion (μm^2) in presence or absence of 50 μM of compound. **(A)** Strain TIGR4, **(B)** Strain D39, **(C)** Strain R6, **(D)** Strain 85, **(E)** Strain 85 *galU* mutant, **(F)** Strain M23, **(G)** Strain M23 *galU* mutant. Error bars represent SD. Asterisks represent statistical differences between control group and treatment groups (Unpaired *t*-test; * $p \leq 0.05$; **** $p \leq 0.0001$) ($n = 150-300$).
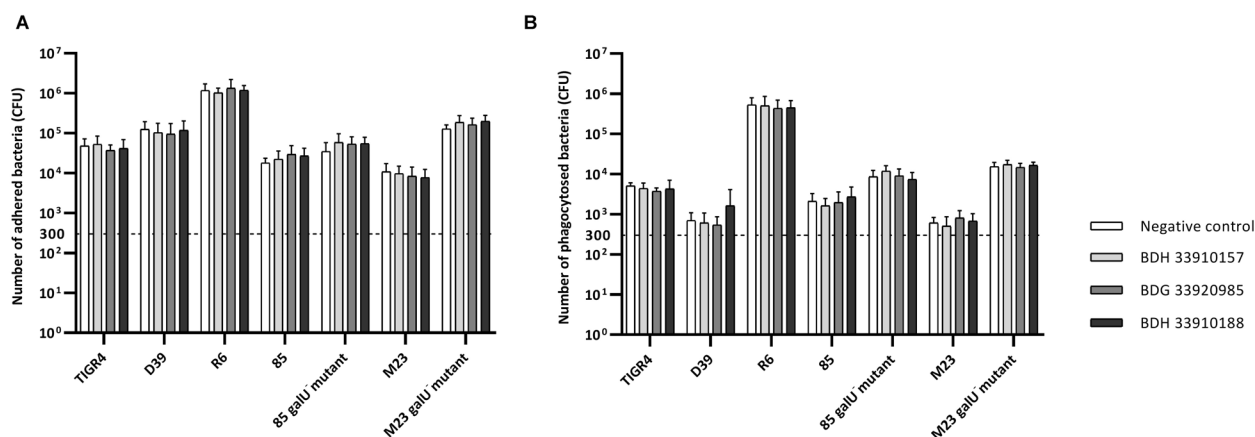
FIGURE 4 | Number of adhered and phagocytosed bacteria with RAW 364.7 macrophage cells. **(A)** Total number of adhered and phagocytosed bacteria, obtained with Triton X-100 treatment, **(B)** Number of phagocytosed bacteria, obtained after removal of extracellular bacteria by gentamicin treatment. No statistical differences between control groups and treatment groups were observed (Two-way Anova) ($n = 3 \times 2$).
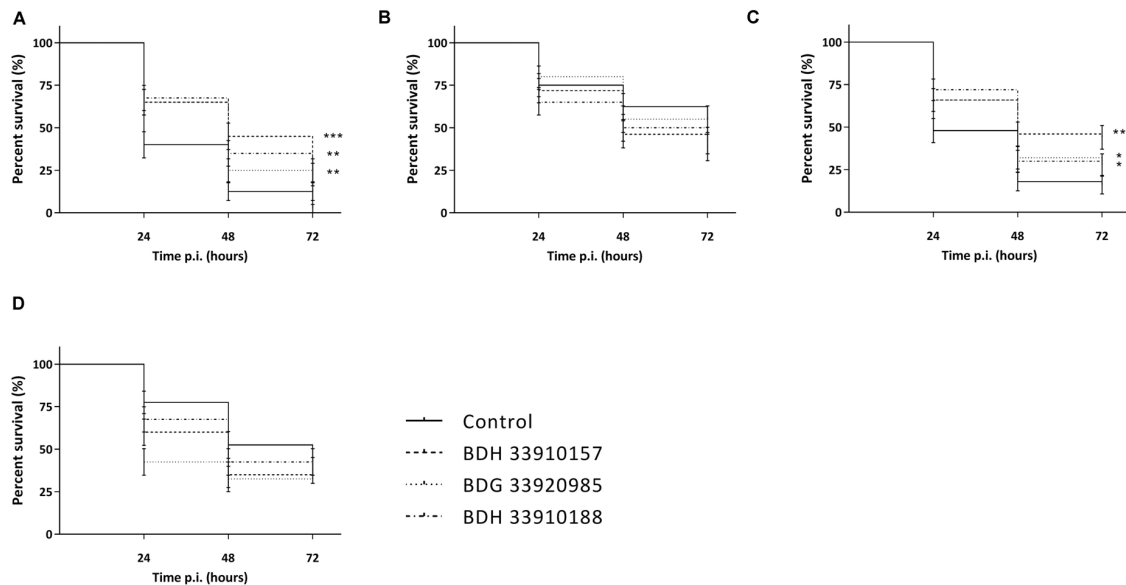
FIGURE 5 | Kaplan-Meier survival curves of *G. mellonella* after infection with *S. pneumoniae* grown in presence or absence of compounds. **(A)** Strain TIGR4, **(B)** Strain D39, **(C)** Strain 85, **(D)** Strain M23. Error bards represent SE. Asterisks represent statistical differences between control group and treatment groups (Survival analysis; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) ($n = 4 \times 10$).
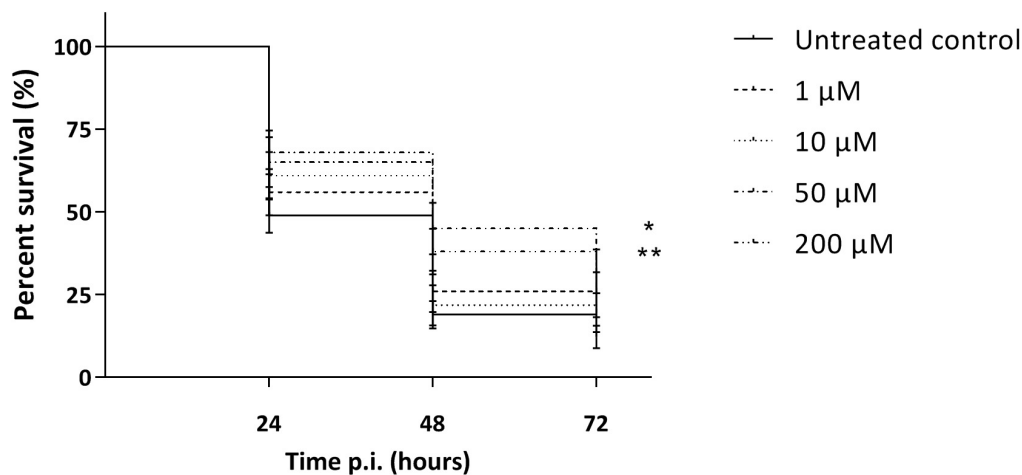
FIGURE 6 | Kaplan-Meier survival curves of *G. mellonella* after infection with *S. pneumoniae* strain TIGR4 grown in presence of 1, 10, 50 or 200 μM of compound BDH 33910157. Error bards represent SE. Asterisks represent statistical differences between control group and treatment groups (Survival analysis; * $p \leq 0.05$, ** $p \leq 0.01$) ($n = 5 \times 10$).

33910157: $p = 0.00330$, strain 85 – BDG 33920985: $p = 0.0640$, strain 85 – BDH 33910188: $p = 0.0211$, Log-rank Mantel-Cox test). This effect coincides with the data of the FITC-dextran exclusion assay, where the compounds were able to reduce bacterial size also in these strains. For strains D39 and M23, where the compounds were not able to reduce bacterial size, the virulence was also not altered (**Figures 5B,D**) (strain D39: $p = 0.3428$, strain M23: $p = 0.3917$, Log-rank mantel-Cox test). Importantly, the increase in bacterial size seen in these strains didn't render them more pathogenic *in vivo*. Lastly, a dose-response curve was obtained for the most active compound, BDH

33910157 (**Figure 6**). Lowering the dose to 1 or 10 μM rendered the compound inactive, while increasing it to 50 or 200 μM significantly improved larval survival compared to an uninfected control group (50 μM: $p = 0.0173$, 200 μM: $p = 0.0033$, Log-rand Mantel-Cox test).

DISCUSSION

While pneumococcal vaccination has proven to be beneficial and has led to a decrease in morbidity and mortality, there

are several downsides. Most importantly, in the years after the introduction of the vaccine a serotype switch to non-vaccine serotypes has been observed (Van Der Linden et al., 2015). Furthermore, herd immunity is only present in young children and not observed in non-vaccine serotypes (Berical et al., 2016; Isturiz et al., 2017). On the other hand, treatment against pneumococcal infections mostly consists of amoxicillin, vancomycin, moxifloxacin or cefuroxime, depending on the antimicrobial susceptibility profile of the infecting agent (Peyrani et al., 2019). While commonly at least one of these antibiotics is capable of battling the pneumococcal infection, antimicrobial resistance is on the rise worldwide and the use of antibiotics should be limited at all cost (McEwen and Collignon, 2018). Therefore, there is a need for novel innovative ways of battling pneumococcal infections, diverging from standard antimicrobial molecules or vaccination strategies. In this study, inhibition of UDPG:PP is proposed and researched as such a novel approach. UDPG:PP is a commonly found enzyme in most life forms, being present in both eukaryotes and prokaryotes (Flores-Díaz et al., 1997; Berbís et al., 2015). Within these domains, UDPG:PP serves a multitude of functions, based on the conversion of UDPG-Glc to Glc-1-P (Mollerach et al., 1998). As glucose plays a pivotal role in a variety of cell processes, UDPG:PP is found to play a role in the integrity of cell membranes, functionality of flagellae, production of LPS and the pneumococcal polysaccharide capsule (Komeda et al., 1977; Deng et al., 2010). While UDPG:PP has previously been proposed as a valid novel drug target, up until recently no inhibitors were known (Kuenemann et al., 2018). In 2018, UDPG:PP inhibitors against listerial UDPG:PP were screened using an *in silico* modeling approach, leading to the identification of several compounds with anti-listerial activity. In *L. monocytogenes*, UDPG:PP is important in the first step of wall teichoic acid galactosylation, which renders the use of antibiotic cefotaxime useless. Addition of UDPG:PP inhibitors elevated the MIC values of this antibiotic drastically, thereby proving UDPG:PP was effectively inhibited (Kuenemann et al., 2018).

In this study, a similar approach to identify pneumococcal UDPG:PP inhibitors was conducted. As the crystal structure of pneumococcal UDPG:PP is currently unknown, the genome of two *S. pneumoniae* strains, TIGR4 and D39, served as a template for the computational part. According to the *in silico* modeling compound BDH 33910157 showed the lowest binding affinity toward *S. pneumoniae* strains TIGR4 and D39. Compound BDG 33920985 only showed a good binding affinity toward strain D39, while BDH 33910188 showed a good binding affinity toward both strains. However, in most biological assays there were no differences between the compounds. Also, while the genome of strains TIGR4 and D39 were both used for the *in silico* modeling, none of the compounds had an effect on strain D39 in a biological setting. To the contrary, a significant increase in bacterial size of strain D39 was seen after incubation with these compounds. This implies *in silico* modeling was effective but not 100% accurate. However, modeling was performed solely based on the primary sequences of these strains without crystal structures of the target. This could explain the contradicting results when using other pneumococcal strains, as small genomic differences could lead to e.g., a significant change in the three-dimensional

conformation of the enzyme, increase or decrease in binding affinity, shielding of the binding place. In *silico* modeling without an actual crystal structure being available is challenging (Kuenemann et al., 2018). While the pneumococcal enzyme has been purified before, the crystal structure remains unidentified (Zavala et al., 2017). However, elucidation of this structure could greatly improve *in silico* modeling and development of novel inhibitors. Currently, the crystal structure of UDPG:PP is only known for several eukaryotes and following bacteria: *Helicobacter pylori* (PDB codes 3JUJ and 3JUK) (Kim et al., 2010), *E. coli* (PDB code 2E3D) (Thoden and Holden, 2007a), *Corynebacterium glutamicum* (PDB code 2PA4) (Thoden and Holden, 2007b), *Acinetobacter baumannii* (PDB codes 6IKX and 6IKZ) (Lee and Kang, 2019), *Sphingomonas elodea* (PDB 2UX8) (Aragao et al., 2007), *Erwinia amylovora* (PDB code 4D48) (Benini et al., 2017), *Yersinia pestis* (PDB code 6MNU) (Gibbs et al., 2019), and *Burkholderia spp.* (PDB codes 5VCT, 5VE7, 5J49, 5I1F) (Abendroth et al., 2016a,b, 2017a,b). Apart from the aforementioned issues with *in silico* modeling based on genetic sequences, biological effects could be lower than expected due to marginal uptake in the cells, rather than poor enzymatic binding. However, in previous research these compounds have proven to be effective against closely related *L. monocytogenes*, implying uptake is possible (Kuenemann et al., 2018).

Several lead compounds were identified *in silico* and a selection of three compounds was tested in subsequent *in vitro* and *in vivo* biological assays. The *G. mellonella* larval *in vivo* model possesses only an innate immune system. This model allowed a better study of the first line of defense and primary recognition, without the interference of an adaptive immunity compared to more complex vertebrate models (Tsai et al., 2016). Overall, the effect of the compounds on bacterial size thus amount of polysaccharide capsule, as seen in the FITC-dextran exclusion assay, was rather limited. While a mutation of UDPG:PP, leading to a dysfunctional enzyme, showed a decrease of approximately 50% in overall size, addition of the compounds decreased the bacterial size by only 10–20%. However, virulent bacterial strains TIGR4 and 85, that showed a statistical decrease in bacterial size – even though small – also showed a clear decrease in *in vivo* virulence. This implies the compounds were capable of inhibiting the UDPG:PP enzyme, which led to at least a partial decrease in pneumococcal polysaccharide capsule and consecutively to a better recognition of the pathogen by the innate immune system. Strains D39 and M23, for which no or an adverse effect of the compounds on bacterial size was observed, showed no differences in *in vivo* virulence. While for strain M23 the polysaccharide capsule size greatly increased (approximately 200%), the innate immune system was able to withstand the infection equally as it did not lead to an increase in *in vivo* virulence. To explain the different effects on bacterial size, the biochemical structure of each serotype provides more insight. Both strains D39 and M23 (respectively, serotypes 2 and 3) incorporate glucuronic acid (GlcA) in their capsules (Geno et al., 2015). UDP-GlcA is readily formed out of UDP-Glc, regulated by UDPG:PP (Mollerach et al., 1998). A decrease in available UDP-Glc and subsequent UDP-GlcA might lead to a less well-organized capsule, looser conformation and thus

increase in overall size. On the other hand, the capsule of strains TIGR4 and 85 (respectively, serotype 4 and 14) doesn't contain GlcA (Geno et al., 2015). Therefore, a shortage in UDP-Glc won't directly influence their capsule but will lead to a general shortage in sugars. On its turn, this could lead to a decrease in overall capsule production. As the compounds don't completely inhibit UDPG:PP, there is no complete abolishment of capsule. If UDPG:PP would be fully inhibited, the shortage in UDP-Glc would be more severe, which would render the bacteria unable to synthetize capsule regardless of serotype, as was seen earlier when using *galU* mutant strains (Cools et al., 2018). It should be noted that bacterial size was also affected in some strains already deficient either of capsule (R6) or of functional UDPG:PP (M23 *galU* mutant). As UDPG:PP serves a multitude of purposes in the prokaryotic cell, it is feasible that another pathway was also affected, especially when there is no capsule present (Berbís et al., 2015). On the other hand, the compounds were not screened for their selectivity against UDPG:PP, thus they might potentially interfere with other enzymes as well, which could also have an effect on capsule formation.

Furthermore, there was no effect of the compounds on planktonic pneumococcal growth nor could a MIC be determined, thus the decrease in virulence could not be attributed to a classic antimicrobial working mechanism. This implies the bacteria encountered no immediate negative consequences of these compounds. More likely, the larval innate immune system got triggered by the decreased amount of capsule, which led to an increase in phagocytosis rate and decrease in virulence, as observed before (Preston and Dockrell, 2008). This sort of therapy is considered a good adjuvant to the conventional antimicrobial therapy in the light of the battle against antimicrobial resistance (Sadgrove and Jones, 2019). Lastly, while the *in vivo* model rendered multiple statistical differences between treated groups and untreated controls, this effect was not seen in *in vitro* adherence to and phagocytosis in a macrophage cell line. However, pneumococcal strains without a functional UDPG:PP enzyme clearly showed an increase in adherence and phagocytosis compared to their non-mutated parent strains (Cools et al., 2018). This again implies that, while the compounds might partially inhibit the enzyme, they are not capable of fully inhibiting it, thus abolishing all polysaccharide capsule. The *in vitro* macrophage assay is probably not sensitive enough to detect these more subtle differences in virulence. Off note, this finding stretches the importance of fast and cheap *in vivo* models, to consolidate or contest *in vitro* data before either discarding compounds or research ideas or moving toward more complex *in vivo* models (Cools et al., 2019).

Several other anti-virulence drug targets against pneumococci have been proposed. Concerning inhibition of polysaccharide capsule, CpsB, a tyrosine phosphatase encoded by *cpsB*, has been suggested as novel drug target, as *cpsB* mutants have been shown to be avirulent in several animal models of infection (Morona et al., 2004; Standish et al., 2012; Monteiro Pedroso et al., 2017). Fascioquinol E – an extract derived from the marine sponge *Fasciospongia spp.*, has been shown to inhibit CpsB phosphatase activity and to increase macrophage adherence *in vitro* (Standish et al., 2012). Other strategies include modification of the bacterial cell wall, inhibition of pneumolysin

and inhibition of quorum sensing. Lysozyme, a component of the human immune system, is known to be important in degradation of bacterial peptidoglycan layers, thereby destabilizing the bacterial cell wall. However, pneumococci can withstand this lysing enzyme by a deacetylation process, catalyzed by peptidoglycan N-acetylglucosamine deacetylase A (PgdA). Mutant pneumococci lacking this enzyme are more susceptible to lysozyme *in vitro* and show a reduction in virulence *in vivo* (Vollmer and Tomasz, 2002). Also, *in silico* inhibitors of PgdA have been described (Bui et al., 2011). Multiple studies have shown that inhibition of pneumolysin, a virulence factors known to be essential for bacterial survival in the respiratory tract, reduces mortality in *in vivo* models (Kadioglu et al., 2008; Arzanlou et al., 2011; Li et al., 2015; Zhao et al., 2016, 2017; Song et al., 2017a,b). Apart from direct inhibition, also sequestration of pneumolysin in liposomes has been shown beneficial on infection outcome in animal models (Henry et al., 2015; Baumgartner et al., 2016). Several antimicrobial peptides, analogs of indolicidin and ranalexin, are also proposed as pneumolysin inhibitors. However, their mechanism of action might also include inhibition of autolysin (Jindal et al., 2015, 2017). Finally, quorum sensing inhibitors have proven to effectively prevent *in vitro* and/or *in vivo* biofilm formation on middle ear implants and migration of pneumococci to the blood (Yadav et al., 2012, 2014, 2015; Cevizci et al., 2015; Motib et al., 2017).

In conclusion, we have shown that UDPG:PP inhibitors possess a great potential in the search for novel anti-virulence modulators. However, elucidating the crystal structure of pneumococcal UDPG:PP would benefit the development of adequate and selective inhibitors. Furthermore, development of future inhibitors should focus on inhibiting pneumococcal UDPG:PP regardless of *S. pneumoniae* serotype, while disregarding other prokaryotic or eukaryotic UDPG:PP. This research is the first report on using UDPG:PP inhibitors against pneumococcal infections and supports the idea of using UDPG:PP as a novel drug target.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FC, PC, and DF conceived the presented idea. FC, NG, and DT carried out the experiments. DT, PD, DF, and PC contributed to interpretation of results. FC and DF wrote the manuscript with support of DT, NG, PD, and PC. DF and PC helped supervise the project. All authors contributed to manuscript revision, read and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01596/full#supplementary-material

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The Demand for New Antibiotics: Antimicrobial Peptides, Nanoparticles, and Combinatorial Therapies as Future Strategies in Antibacterial Agent Design

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The inappropriate use of antibiotics and an inadequate control of infections have led to the emergence of resistant strains which represent a major threat to public health and the global economy. Therefore, research and development of a new generation of antimicrobials to mitigate the spread of antibiotic resistance has become imperative. Current research and technology developments have promoted the improvement of antimicrobial agents that can selectively interact with a target site (e.g., a gene or a cellular process) or a specific pathogen. Antimicrobial peptides and metal nanoparticles exemplify a novel approach to treat infectious diseases. Nonetheless, combinatorial treatments have been recently considered as an excellent platform to design and develop the next generation of antibacterial agents. The combination of different drugs offers many advantages over their use as individual chemical moieties; these include a reduction in dosage of the individual drugs, fewer side effects compared to the monotherapy, reduced risk for the development of drug resistance, a better combined response compared to the effect of the individual drugs (synergistic effects), wide-spectrum antibacterial action, and the ability to attack simultaneously multiple target sites, in many occasions leading to an increased antibacterial effect. The selection of the appropriate combinatorial treatment is critical for the successful treatment of infections. Therefore, the design of combinatorial treatments provides a pathway to develop antimicrobial therapeutics with broad-spectrum antibacterial action, bactericidal instead of bacteriostatic mechanisms of action, and better efficacy against multidrug-resistant bacteria.

Keywords: ESKAPE, MDR, XDR, antimicrobial peptides, metal nanoparticles, combinatorial treatments

INTRODUCTION

Development of antibacterial resistance is considered one of the leading public health problems, since it has a significant impact on the economy worldwide. Since therapeutic options to treat infections are increasingly being limited due to antibacterial resistance, this escalates the morbidity and mortality associated with infectious diseases caused by bacteria [World Health Organization (WHO), 2020]. ESKAPE

pathogens are responsible for the majority of life-threatening nosocomial infections and are capable of "escaping" the biocidal action of antimicrobial agents (Pendleton et al., 2013). The term "ESKAPE" is an acronym for six bacterial pathogens associated with multidrug resistance: *Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Enterobacter spp.* (Mulani et al., 2019). Multidrug-resistant (MDR) bacteria are resistant to more than one antimicrobial drug, and extensively drug-resistant (XDR) bacteria are types of drug-resistant organisms that are resistant to all, or almost all, approved antimicrobial agents (Magiorakos et al., 2012). For these reasons, it is essential to design and engineer new promising classes of antibiotics (Gajdács, 2019).

THE NEW THERAPEUTIC ALTERNATIVES: INPUT FROM RECENT STUDIES

As we described above, the development of antimicrobial resistance represents a major threat to public health, and this has been echoed by different health organizations around the globe. Antimicrobial peptides (AMPs) and nanoparticles (NPs) and the design of novel combinatorial therapies are among the new promising alternatives to fight infections caused by MDR- and XDR-resistant bacteria.

Antimicrobial Peptides

Antimicrobial peptides are a highly diverse family of small proteins with a varying number of amino acids; they have also been referred to as cationic host defense peptides (Boparai and Sharma, 2019). A variety of synthetic AMPs have been synthesized in the laboratories, but there are also a wide diversity of AMPs produced by bacteria and yeast, in addition to those found naturally in animals and plants (Wang, 2013). AMPs have demonstrated to participate in a variety of biological activities, including as antimicrobial antiviral, antifungal, and anti-mitogenic agents, in addition to their antitumor and anti-inflammatory properties and their ability to act as immune modulators. Therefore, AMPs represent a potential alternative to replace a wide variety of commonly used drugs. Moreover, most of the available studies demonstrate that AMPs exhibit therapeutic activity in *in vitro* and *in vivo* models (Divyashree et al., 2019).

The use of AMPs alone or in combination with conventional drugs has proven effective in combating different infectious agents, mainly MDR bacteria (Zharkova et al., 2019). AMPs are promising potential candidates to counteract multiresistant pathogens since they possess many advantages: they display potent microbicidal activity in the micromolar range (Aoki and Ueda, 2013), they have demonstrated a rapid bacterial death action (Lei et al., 2019), and they have low resistance selection (Mahlapuu et al., 2016). Their mechanism of antibacterial action is multifunctional because it alters the cell membrane (Li et al., 2017) and also attacks specific targets that take part in

the development of different intracellular processes (Le et al., 2017), such as inhibition of transcription, translation, protein synthesis, and bacterial cell wall formation (Mwangi et al., 2019). These general mechanisms of action of AMPs are displayed in **Figure 1A**.

One AMP of particular interest is human cathelicidin peptide (LL-37), which has been reported to have wound-healing effects on the host in addition to exhibiting antimicrobial and anti-biofilm activity against a variety of Gram-positive and Gram-negative human pathogens (Duplantier and van Hoek, 2013). LL-37 and its derivatives are considered excellent candidates as antimicrobial therapeutic agents and have been the subject of many studies (Dürr et al., 2006; Kościuczuk et al., 2012; De Breij et al., 2018). Especially, in 2018, De Breij et al. synthesized an LL-37 derivative (SAAP-148), with potent antimicrobial activities, by replacing an amino acid from the terminal carbon of the LL-37 chain. This LL-37 derivative exhibited a minimum inhibitory concentration [MIC] between 0.4 and 12.8 μM against various ESKAPE pathogenic bacteria (e.g., *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) without selection of resistance. Furthermore, this AMP derivative showed anti-biofilm activity against *S. aureus*, *A. baumannii*, and *P. aeruginosa* (De Breij et al., 2018).

Colistin is another important peptide antibiotic (produced *Bacillus polymyxa* var. *colistinus*) used as a last-resort drug to treat MDR infections (Oka and Ito, 2000). It has emerged as an important agent in the treatment of Gram-negative bacterial infections, especially those caused by MDR pathogens in hospitalized patients (Das et al., 2017). Notably, two new colistin-derived AMPs (AA139 and SET-M33), with a mechanism similar to colistin, are in development and have shown excellent therapeutic potential both *in vitro* against MDR bacteria and in *in vivo* infection models (van der Weide et al., 2019).

The main limiting factor for the systemic use of AMPs is their sensitivity to proteolytic digestion in different body fluids (e.g., intestinal mucosa, gastrointestinal tract, and blood plasma), which directly affect both their *in vivo* stability and their pharmacokinetic profile (Moncla et al., 2011; Starr and Wimley, 2017). Therefore, the search for new AMPs continues, particularly in a new class of peptides with high specificity and potency, known as "selectively targeted AMPs" (STAMPs), which show increased sensitivity to specific pathogens, demonstrating a significant increase in their bactericidal capacity without direct effects on the microbiota (Chung and Khanum, 2017). The STAMP technology requires two functionally independent peptide domains integrated through a small linker. One peptide domain serves as the killing AMP moiety and the other peptide domain consisting of a high-affinity binding peptide which functions as a targeting moiety (Aoki and Ueda, 2013). These properties increase the binding to the surface of a targeted pathogen by enhancing the local concentration of the AMP and thus lead to improve bactericidal efficiency (Sarma et al., 2018). In recent years, several new and promising STEMs have been developed against *Streptococcus mutans* (Huo et al., 2017), *Pseudomonas aeruginosa*, and *Streptococcus mutants* together (He et al., 2009), methicillin-resistant *Staphylococcus aureus* (Mao et al., 2013), *Enterococcus faecalis* (Xu et al., 2020), and

FIGURE 1 | Mechanism of antimicrobial action in bacteria: **(A)** General mechanisms of action of antimicrobial peptides (AMPs). AMPs act through different mechanisms (Le et al., 2017) such as 1. Alteration in membrane integrity via electrostatic interaction with negatively charged cell membranes which kill cells, 2. Inhibition of DNA synthesis by (i) cross-linking with single- or double-stranded DNA, (ii) prevention of the DNA relaxation by inactivation of DNA topoisomerase I, and (iii) blocking of DNA replication by trapping the gyrase-DNA complex; and protein synthesis by (i) inhibition of protein translation by targeting the ribosomes, (ii) interrupting the protein-folding pathway, and (iii) rapid proteolytic activity causing the degradation of some DNA replication-associated proteins, leading to secondary inhibition of DNA synthesis, 3. Inhibition of bacterial cell wall formation by alteration of the alternating amino sugars in linear form that cross-link via peptide bridges to form the peptidoglycan layer, and 4. Inhibition of metabolic pathways by alteration of nucleic acid metabolism, including nucleotide transport and metabolism, nucleobase, nucleoside, and nucleotide interconversion. **(B)** Mechanisms for antimicrobial action of metal nanoparticles (MNPs). MNPs act via the following (Shaikh et al., 2019). 1. MNPs disturb cell membrane permeability by interfering with metabolic pathways and inducing changes in membrane shape and function. 2. When MNPs are in solution, metal ions are released in the environment surrounding. Metal ions generate reactive oxygen species (e.g., oxygen ions and hydroxyl radicals) and induce oxidative stress in bacteria. Oxidative stress is a key contributor in altering the bacterial membrane permeability and thus can damage cell membranes. Also, metal ions may cause cell structural changes and aberrant enzyme activities, which perturb normal physiological processes. 3. Interaction with sulfur- and phosphorous-containing compounds such as DNA, which prevent DNA from unwinding and transcription.

clinical isolates (*Pseudomonas aeruginosa*; Eckert et al., 2006). Nonetheless, more preclinical and clinical research is needed in the development of targeted antimicrobial therapy.

Metal Nanoparticles

An additional alternative to fighting infections caused by antibiotic-resistant bacteria is the development of NPs since it has been amply reported that metal nanoparticles (MNPs) have antibacterial activity against ESKAPE pathogens (Wang et al., 2017; Lee et al., 2019). Some of the mechanisms of the antimicrobial mode of action of MNPs are summarized in **Figure 1B**. In the search for new antimicrobials to treat the ESKAPE pathogens, silver has been highlighted as a potential candidate to treat infectious diseases (Borthagaray et al., 2018). Silver nanoparticles (AgNPs) possess antimicrobial activity, and they act by disturbing cell membrane permeability,

interacting with sulfur- and phosphorous-containing compounds including DNA, in addition to their ability to release silver ions, contributing to the antibacterial effect (Morones et al., 2005; Morones-Ramirez et al., 2013). Gold (Au) nanoparticles have also been reported as effective antibacterial agents for antibiotic-resistant bacterial strains such as *S. aureus*, *E. faecium*, *Enterococcus faecalis* (*E. faecium*), *Escherichia coli* (*E. coli*), *Vibrio cholerae* (*V. cholerae*), *Salmonella typhimurium* (*S. typhimurium*), and *Salmonella dysenteriae* (*S. dysenteriae*; Kumar et al., 2016).

Among metal oxide nanoparticles, zinc oxide (ZnO) nanoparticles have shown antimicrobial activity against both Gram-negative and Gram-positive bacteria, including *Bacillus subtilis* (*B. subtilis*), *S. aureus*, *E. coli*, *P. aeruginosa*, and *A. baumannii* (Guo et al., 2015; Tiwari et al., 2018). On the other hand, among photocatalytic nanoparticles, titanium dioxide (TiO$_2$) NPs have been extensively studied due to their

antimicrobial activity (Gelover et al., 2006). Several studies have reported the antimicrobial activity of TiO$_2$ NPs against methicillin-resistant *S. aureus* and MDR *E. coli* (Jesline et al., 2015; Mantravadi, 2017; de Dicastillo et al., 2019).

Despite the advantages that nanoparticles offer, such as a broad therapeutic index, controlled drug release, less prone to bacterial resistance, and fewer side effects than chemical antimicrobials (Lee et al., 2019), to treat infections caused by the ESKAPE pathogens, there are still challenges remaining to be tackled such as improvement of physicochemical properties, better pharmacokinetic profiles, and comprehensive studies on long-term exposure to humans. In terms of design and application, there is a particular interest in the generation of nanohybrids combining different metals with different antimicrobial and sensitizer agents (Zhang et al., 2014; Wolfram et al., 2015). Metal nanoparticle-based compounds (alone or in combination with other antimicrobial agents) provide promising alternatives to combat the development of antibacterial resistance (Shaikh et al., 2019). Therefore, it is imperative to develop a comprehensive understanding of the mechanisms of action responsible for the bactericidal properties as well as the identification of the most promising antimicrobial agents for future clinical translation.

Combinatorial Treatments

The strategies to reduce antibiotic resistance include the limited use of antibiotics and the application of more effective antibacterial therapies. Because the time of exposure to antibiotics correlates with the development of resistance (Andersson et al., 2020), it is necessary to use drugs with a broad spectrum of action and pharmacokinetic properties that facilitate their rapid access to the target site (Krause et al., 2016). However, most of the available treatments do not have all these characteristics, so an alternative option is the use of combination therapies, which can lead to a synergistic and more effective response (Lehár et al., 2009). It has been shown that the combination of drugs leads to a considerably more potent effect, compared to the individual drug (Tamma et al., 2012; Marks et al., 2013). **Figure 2** displays the disadvantages of using single drugs (**Figure 2A**) and the advantages of using combinatorial treatments (**Figure 2B**).

Antimicrobial Peptide-Based Combinatorial Treatments

Combinations of AMPs with antibiotics have been reported to show synergistic effects in the treatment of bacterial infections. The mechanism of antibacterial action in these combinations involves the disruption of the outer membrane (Cassone and Otvos, 2010). Moreover, the use of AMPs in combinatorial treatments has certain advantages over their use as a single treatment since it has been observed that in combinatorial treatments, AMPs work as enhancers of the antimicrobial effects. This characteristic allows the reduction of their dose, and it also unlocks the bactericidal application of molecules with low molecular weight, which typically do not exhibit antimicrobial properties (Si et al., 2020).

Recent studies have demonstrated the synergistic activity of antibiotics combined with AMPs. Akbari et al. (2019) reported the synergism and other drug interactions between melittin, a cationic amphipathic peptide, and antibiotics such as doripenem, doxycycline, colistin, and ceftazidime, against MDR isolates of *A. baumannii* and *P. aeruginosa*. Likewise, combinatorial treatments of conventional antibiotics with new synthetic peptides inspired by human cationic peptides LL-37 and thrombocidin-1 (TC-1) have shown synergistic activity against *S. aureus* (antibacterial and anti-biofilm activity; Koppen et al., 2019). In addition, the synergistic activity of 30 short AMPs combined with several conventional antibiotics such as beta-lactam antibiotics, cephalosporins, aminoglycosides, and quinolones was tested against an MDR *P. aeruginosa* isolate (PA910; Ruden et al., 2019). Several combinations between peptides, polymyxin B, erythromycin, and tetracycline, as well as novel variants of indolicidin were found to be synergistic. Furthermore, the results showed that a single amino acid substitution within the peptides can have a powerful effect on the ability to synergize, which represents an opportunity to design treatment strategies based on synergistic interactions (Ruden et al., 2019).

Metal Nanoparticle-Based Combinatorial Treatments

Metal nanoparticles should be considered as an attractive alternative to potentiate the antimicrobial effect of old and current antibiotics, since they have a high tendency to act synergistically when combined with a wide variety of antibiotics (Bankier et al., 2019). This, in addition to the increased biocompatibility achieved by synthesizing them through green chemistry, allows considering the use of MNPs as adjuvant agents for the treatment of infectious diseases (Rout et al., 2018).

In the past years, there has been a marked increase in the use of biopolymers (e.g., proteins, nucleic acids, and polysaccharide) as capping agents to functionalize and stabilize MNPs (Sharma et al., 2019). Exopolysaccharides are biocompatible and eco-friendly biomolecules; therefore, they can be used in the synthesis of MNPs (Escárcega-González et al., 2018). Recently, a silver-based nanobiocomposite was synthesized using an exopolysaccharide produced by *Rhodotorula mucilaginosa* UANL-001L (EPS). The results showed an increased antibacterial and anti-biofilm activity of this nanobiocomposite against pathogens of clinical relevance (Vazquez-Rodriguez et al., 2020). Moreover, nanocomposites have been synthesized through green chemistry, such as zinc (Zn) and nickel (Ni) MNPs capped with EPS as capping agents, and they have displayed interesting antimicrobial properties as well. Ni-EPS nanoparticles exhibited both antimicrobial and anti-biofilm activity against resistant MDR strains of *S. aureus* and *P. aeruginosa*. Furthermore, Zn-EPS nanoparticles showed antimicrobial activity for treatments against MDR *S. aureus* and *P. aeruginosa* (Garza-Cervantes et al., 2019).

Among the most studied nanomaterials are silver nanoparticles due to their antimicrobial activity against Gram-positive and Gram-negative bacteria. They can be used in combinatorial treatments with currently used antibiotics for enhanced antimicrobial activity (Shahverdi et al., 2007; Kora and Rastogi, 2013; Naqvi et al., 2013; Singh et al., 2013; Panácek et al., 2016; Lopez-Carrizales et al., 2018; Perveen et al., 2018; Vazquez-Muñoz et al., 2019). Nonetheless, some other MNPs
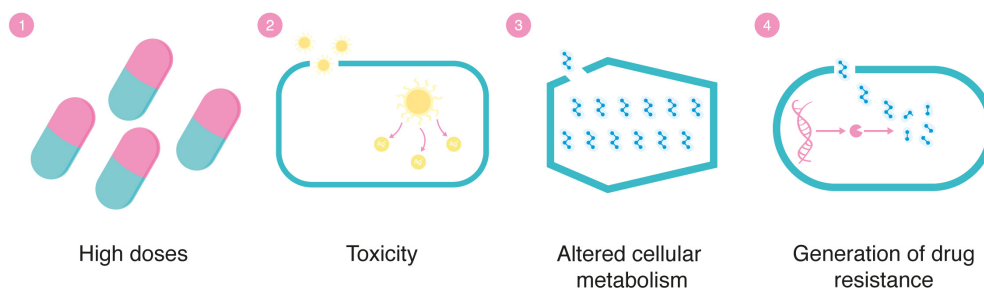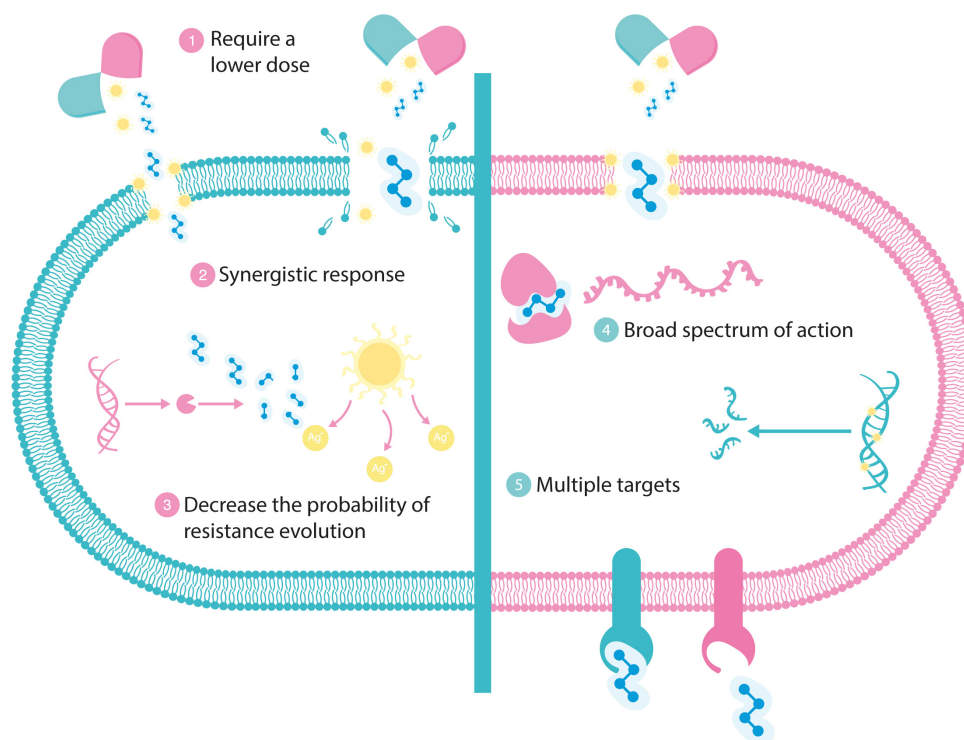
FIGURE 2 | Antimicrobial treatment strategies: **(A)** disadvantages of using single drugs and **(B)** advantages of using combinatorial treatments. The advantage of using combinatorial treatments of synergistic drug pairs provides the opportunity to lower the dosage of the individual agents, thereby reducing toxicity while maintaining the wanted effect on bacteria. Moreover, a synergistic response can occur because of complementary drug action (multiple targets sites on the same protein or pathway are hit; Pemovska et al., 2018). By combining two drugs that achieve the same effect through different mechanisms of action, the development of resistance to a single drug in the combination may be less likely to occur, and when it does occur, it may have a lower impact on the therapeutic outcome (Pirrone et al., 2011). Finally, the use of more than one agent broadens the antibacterial spectrum of the empirical therapy and thus ensures that at least one agent will cover the infecting organism (Gurjar et al., 2014).

such as gold (El-Sheekh and El Kassas, 2014; Kalita et al., 2016; Al-Mawlawi and Obaid, 2019; Arya et al., 2019; Lee and Lee, 2019; Nishanthi et al., 2019; Yang et al., 2019), copper (Khurana et al., 2016; Woźniak-Budych et al., 2017; Murugan, 2018; Selvaraj et al., 2019), and zinc (Banoee et al., 2010; Bhande et al., 2013) have been used in combination with a variety of antibiotic families to enhance bactericidal efficacy.

Some other interesting studies of silver-based nanomaterials have been reported. A novel silver-microfibrillated cellulose biocomposite has been synthesized, and its antimicrobial activity was determined against relevant clinical strains. The results showed that this biocomposite has antimicrobial activity against Gram-negative and Gram-positive bacteria so that it could be applied in the development of biocompatible biomedical devices

TABLE 1 | Antimicrobial peptides, metal nanoparticles, and combinatorial treatments: mechanism of action, tested bacterial strains, advantages, and disadvantages.

	Mechanism of action	Tested bacterial strains	Advantages	Disadvantages
Antimicrobial peptides (AMPs)	1. Alteration in membrane integrity. 2. Inhibition of DNA and protein synthesis. 3. Inhibition of bacterial cell wall formation. 4. Inhibition of metabolic pathways.	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, multidrug-resistant strains.	1. Show potent microbicidal activity in the micromolar range. 2. Rapid bacterial death action. 3. Low resistance selection.	1. High sensitity to proteolytic digestion in different body fluids. 2. Low *in vivo* stability. 3. Reduced pharmacokinetic profile.
Metal nanoparticles (MNPs)	1. Disruption of cell membrane and increased permeability. 2. Releasing metal ions. 3. Interaction with DNA	*Enterococcus faecium*, *Enterococcus faecalis, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Escherichia coli, Salmonella typhimurium, Salmonella dysenteriae, Vibrio cholerae, Bacillus subtilis,* multidrug-resistant strains.	1. Broad therapeutic index. 2. Controlled drug release. 3. Less prone to bacterial resistance. 4. Fewer side effects than chemical antimicrobials.	1. Need to improve metal ions release from MNPs. 2. Moderate stability in biological fluids. 3. Reduced long-term toxicity studies.
Combinatorial treatments	1. Synergistic response. 2. Multiple cellular targets for antimicrobial action. 3. Combination of bactericidal and bacteriostatic mechanism of action.	*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Escherichia coli Mycobacterium tuberculosis* multidrug-resistant strains.	1. Require lower dose than a single drug. 2. Reduced toxicity. 3. Synergisms and more effective response. 4. Decrease the probability of resistance evolution. 5. Better efficacy against multidrug-resistant bacteria.	1. Physical-chemical compatibility among antimicrobial agents. 2. Possible pharmacokinetic and pharmacodynamic interactions.

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(Garza-Cervantes et al., 2020a). Another promising approach toward the development of new antimicrobial combinatorial treatments is the use of transition metals, since they exhibit rapid and significant toxicity, at low concentrations, in different bacterial strains. Garza-Cervantes et al. evaluated the synergistic antimicrobial effects of silver/transition-metal combinatorial treatments. Their results showed combinatorial treatments that exhibited synergism (Ag–Zn, Ag–Co, Ag–Cd, Ag–Ni, and Ag–Cu) since their antimicrobial effects are increased up to 8-fold, compared to the effects observed for the treatments with the individual metals (Garza-Cervantes et al., 2017). Furthermore, Montelongo-Peralta et al. reported synergism between transition metals and antibiotics used to treat first-line drug-resistant strains of *Mycobacterium tuberculosis* (*M. tuberculosis*). Combinatorial treatments composed of isoniazid/silver exhibited a synergistic bactericidal effect in an isoniazid-resistant clinical strain of *M. tuberculosis* (Montelongo-Peralta et al., 2019).

Moreover, a previous study showed the ability of silver to potentiate the activity of a broad range of antibiotics against Gram-negative bacteria, as well as to restore antibiotic susceptibility (re-sensitizing) to a resistant bacterial strain (Morones-Ramirez et al., 2013). Recently, a group of researchers achieved to re-sensitize antibiotic-resistant *E. coli* using transition-metal micronutrients (Cu2+, Zn2+, Co2+, Cd2+, and Ni2+) combined with antibiotics (ampicillin and kanamycin). These combinatorial treatments showed a therapeutic activity and no toxicological effects in a murine topical infection model caused by antibiotic-resistant strains (Garza-Cervantes et al., 2020b). The above data therefore strongly suggest that combination therapies are a potential strategy in the development of new treatments against infectious diseases.

The search for a new generation of antimicrobials to mitigate the spread of antibiotic resistance is urgent (de la Fuente-Nunez et al., 2017). Current research and technology developments have promoted the improvement of antimicrobial agents that selectively target a target site (e.g., a gene, a cellular process, or a specific pathogen; de la Fuente-Nunez et al., 2017; Jackson et al., 2018). AMPs and MNPs exemplify a novel approach for treating infectious diseases. Nonetheless, the combinatorial treatments are considered as an excellent option for designing and developing next-generation antibacterial agents. As summary, **Table 1** describes the mechanism of action, tested bacterial strains, advantages and disadvantages of AMPs, MNPs, and combinatorial treatments.

The selection of appropriate combinatorial treatment is critical for the successful prevention of infections (Bayramov and Neff, 2017). The most important challenges include (i) selection of agents with ideal physical–chemical properties (hydrosolubility and chemical stability in biological fluids; Ebejer et al., 2016), (ii) selection of antimicrobials that display appropriate pharmacokinetics and pharmacodynamics properties (Preston, 2004), (iii) selection of biocompatible capping agents or biopolymer-based materials that enable drug release (Campoccia et al., 2013), and (iv) development of a process that ensures the stability and does not compromise the performance of the combination therapy formulation as a whole (Wu and Grainger, 2006). Therefore, the design of combinatorial treatment provides a pathway to develop antimicrobial therapeutics with broad-spectrum antimicrobial activity, bactericidal instead of bacteriostatic mechanism of action, and better efficacy against MDR bacteria.

AUTHOR CONTRIBUTIONS

Conceptualization, AL-B and JM-R. Writing-original draft preparation AL-B, CG-C, JG-C, JL-E, and JM-R. Graphic design, JL-E. Writing-review and editing, AL-B, CG-C, JG-C, and JM-R. Supervision, AL-B and JM-R. All authors contributed to the article and approved the submitted version.

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2,4-Di-Tert-Butylphenol Isolated From an Endophytic Fungus, *Daldinia eschscholtzii*, Reduces Virulence and Quorum Sensing in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is among the top three gram-negative bacteria according to the WHO's critical priority list of pathogens against which newer antibiotics are urgently needed and considered a global threat due to multiple drug resistance. This situation demands unconventional antimicrobial strategies such as the inhibition of quorum sensing to alleviate the manifestation of classical resistance mechanisms. Here, we report that 2,4-di-tert-butylphenol (2,4-DBP), isolated from an endophytic fungus, *Daldinia eschscholtzii*, inhibits the quorum-sensing properties of *P. aeruginosa.* We have found that treating *P. aeruginosa* with 2,4-DBP substantially reduced the secretion of virulence factors as well as biofilm, and its associated factors that are controlled by quorum sensing, in a dose-dependent manner. Concomitantly, 2,4-DBP also significantly reduced the expression of quorum sensing-related genes, i.e., *lasI*, *lasR*, *rhlI*, and *rhlR* significantly. Importantly, 2,4-DBP restricted the adhesion and invasion of *P. aeruginosa* to the A549 lung alveolar carcinoma cells. In addition, bactericidal assay with 2,4-DBP exhibited synergism with ampicillin to kill *P. aeruginosa.* Furthermore, our computational studies predicted that 2,4-DBP could bind to the *P. aeruginosa* quorum-sensing receptors LasR and RhlR. Collectively, these data suggest that 2,4-DBP can be exploited as a standalone drug or in combination with antibiotic(s) as an anti-virulence and anti-biofilm agent to combat the multidrug resistant *P. aeruginosa* infection.

Keywords: anti-quorum sensing, 2,4-Di-tert-butylphenol, endophytic fungi, multidrug resistance, *P. aeruginosa*

INTRODUCTION

Quorum sensing is a bacterial signaling mechanism through which bacteria sense their cell density and activate a range of coordinated behaviors once their population reaches a threshold (Rutherford and Bassler, 2012). Bacteria release signaling molecules, called autoinducers, which accumulate as the cell density of the bacteria increases. QS regulates an array of bacterium physiological

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Abbreviations: 2,4-DBP, 2,4-Di-tert-butylphenol; BAC, baicalein; MIC, minimum inhibitory concentration; QS, quorum sensing.

activities, such as virulence, pathogenesis, biofilm formation, swimming, and swarming motility. Since several of these functions are central to bacterial persistence and pathogenesis, QS has been regarded as an attractive target for anti-biofilm and anti-QS-based alternative anti-microbial therapy. However, little progress has been made concerning QS-based alternative anti-microbial therapies. *Pseudomonas aeruginosa*, well known to be an opportunistic, notorious nosocomial pathogen responsible for causing a range of acute and chronic infections, such as respiratory tract infections, urinary tract infections, infections in the central nervous system, and skin and soft tissue infections in immuno-compromised patients (Bjarnsholt et al., 2010). *P. aeruginosa* engages in QS by three independent, but by cross-talking, LasR-LasI, RhlR-RhlI, and PQS-PqsR QS signaling systems, where the autoinducer for the LasR-LasI system is N-(3-oxo-dodecanoyl) homoserine lactone (3OC$_{12}$ HSL), and the RhlR-RhlI system utilizes C4 (butanoyl) HSL (Pearson et al., 1995). The LasRI system of this bacterium regulates the expression of several genes encoding various virulence factors (Schuster et al., 2003). The autoinducer of the third QS system PQS-PqsR, 2-heptyl-3-hydroxy-4(1H) quinolone (PQS), binds to the transcriptional regulator PqsR and further controls downstream targets, including biofilm formation, which leads to antibiotic tolerance and resistance without the need for specific antibiotic inactivating enzymes. Therefore, QS inhibitors could have this dual advance of rendering the bacterium non-virulent and sensitizes it toward antibiotics. QS inhibitors are anticipated to curtail the pathogenicity, since the expression of several virulence factors and the facilitation of a successful infection are under QS regulation (Pearson et al., 2000). Hence, molecules interfering QS could be an aid to the existing armamentarium against *P. aeruginosa* infections. These strategies include degrading AHL molecules enzymatically by acylases, lactonases, and oxidoreductases, outcompeting/inhibiting QS signal molecules by structurally similar inhibitory molecules to bind to their cognate regulatory proteins, or by quorum quenching antibodies and macromolecules such as cyclodextrins that scavenge autoinducers (Rémy et al., 2018; Ahmed et al., 2019). In addition, several natural substances with known biological properties act as QS inhibitors as they intervene in QS-associated pathways, attenuate QS gene expression, and impair the infection. Recently, several reports claim to quench QS or ameliorate the QS signals through various synthetic molecules, natural products, and enzymes (Fong et al., 2018). For instance, acyl homoserine lactone analogs such as N-acyl cyclopentyl amines (Cn-CPAs), lactonase SsoPox, N-acylhomoserine lactonase, and AiiM (Guendouze et al., 2017; López-Jácome et al., 2019) were effective as QSIs against *P. aeruginosa*. Curcumin and coumarin were reported to inhibit the virulence and biofilm-forming ability of *P. aeruginosa*, while naringenin and taxifolin were reported to reduce the expression of QS-related genes. Furthermore, enzymes such as AHL-lactonases are reported to degrade 3OC8HSL of *P. aeruginosa* and affect the virulence capability and biofilm-forming ability (Kalia et al., 2018).

Recently, we reported that metabolites from endophytic fungi associated with *Carica papaya* also attenuate in *P. aeruginosa*

(Mishra et al., 2018; Meena et al., 2019). Besides being ecologically and physiologically diverse, endophytic fungi are diverse in synthesizing chemically potent and varying secondary metabolites when in association with a medicinally important host (Rashmi et al., 2019). In the present study, we report 2,4-DBP as a QS inhibitor that was isolated from the endophytic fungi *Daldinia eschscholtzii* associated with host plant *Tridax procumbens*, which is known for its traditional medicinal values (Mir et al., 2017). 2,4-DBP not only impeded QS-mediated virulent factors and biofilm formation but also showed synergistic effects with therapeutically relevant antibiotics. Finally, *in silico* analyses showed it to be an effective QS inhibitor and comparable to the known anti-QS inhibitor BAC.

MATERIALS AND METHODS

Organisms and Reagents

Chromobacterium violaceum ATCC 12472 and *Pseudomonas aeruginosa* PAO1 are the test strains used in the study. Cultures were maintained in Luria–Bertani (LB) broth and routinely subcultured. BAC standard (Sigma-Aldrich, United States) was dissolved in dimethyl sulfoxide (Merck) and was sterilized using a 0.22-μm PVDF membrane filter. Chitin azure, azocasein, and elastin congo red were procured from Sigma-Aldrich (Sigma-Aldrich, United States), Maxima H Minus Reverse Transcriptase from Thermo Scientific, and FastStart Universal SYBR Green Master Mix from Roche (USA). The A549 lung epithelial cell carcinoma cell line was procured from the National Center for Cell Sciences, India, for *in vitro* infection studies.

Isolation and Screening of Potential Endophytic Fungi

Green and healthy leaves of the host *Tridax procumbens* were collected from the Pondicherry University campus, India, 12.0219° N, 79.8575° E. After surface sterilization according to Cui et al. (2015), endophytic fungi were isolated, subcultured, and maintained as axenic cultures. All the endophytic fungi isolated were screened for their anti-QS potential against *Chromobacterium violaceum* (ATCC 12472) and *Pseudomonas aeruginosa* PAO1. Agar well diffusion method was performed to treat the fungal crude samples to a lawn of bacteria and examine the zone of inhibition. The isolate with the largest zones of inhibition was considered as most effective and selected for the rest of the work. The most potent isolate was further subjected to purification of the QS inhibitor compound as described below.

Identification and Phylogenetic Analysis of Selected Endophytic Fungi

After screening the isolates, isolate TP2-6 (an in-house code) was selected for further study. The morphological details of the fungi were observed under stereo-zoom microscope and compound microscope for various characteristics of colony and spore formation (Mishra et al., 2018). For molecular identification, the nuclear ribosomal internal transcribed spacer (ITS) region was amplified by the primers ITS1 (5′-TCC GTA GGT GAA CCT

GCG G-3′) and ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and sequenced by capillary sequencing, followed by phylogenetic analysis as described in Devadatha et al. (2018).

Large-Scale Fermentation of *Daldinia eschscholtzii* and Extraction of Crude Extract

The endophytic fungal isolate *Daldinia eschscholtzii* (TP2-6), which showed the most potent anti-QS activity, was selected for the purification of the active compound responsible for the anti-QS activity against *P. aeruginosa*. About 20 liters of potato dextrose broth (PDB) was used to cultivate *D. eschscholtzii*. From the axenic culture of *D. eschscholtzii*, a loop full inoculum was inoculated into PDB and it was kept for growth for 20 days at 28°C at constant agitation. The fungal broth, after separating the mycelium, was extracted twice with double the volume of ethyl acetate. The organic phase was separated and vacuum dried to obtain the fungal crude extract.

Column Chromatography

The concentrated crude sample of *D. eschscholtzii* was further subjected to column chromatography for purification in a glass column (700 × 30 mm). The glass column was packed with silica gel (60–120 mesh size, Merck) as the stationary phase. A dried powdered crude extract mixed with silica gel/powder (200 mesh size) at a ratio of 1:3 was loaded onto a column as the sample bed. The column was first eluted with hexane, followed by a hexane: ethyl acetate mixture with a gradual increase in polarity in different ratios (9:1, 8:2, 7:3, 6:4, 5:5, etc.), and finally eluted by using 100% of ethyl acetate, methanol, and water. Different fractions were collected and assessed for their anti-QS activity.

High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography of the active fraction was done on an RP-C18 column using photodiode array detectors (PDA-SPD-M20A). The injection volume and flow rate used were 10 μL and 0.50 mL/min, respectively. Acetonitrile along with HPLC-grade water was used as the mobile phase solvent. The elution program of compounds started with 15% acetonitrile reaching up to 100% in 40 min with a hold on this condition for 5 min, and again gradient coming down to 15% acetonitrile in 8 min which was finally held for 5 min (Sharma et al., 2017). The samples and mobile phase were filtered through a 0.2-μm nylon membrane filter before applying into the column. Samples were analyzed at 280 nm wavelength.

Characterization and Structure Analysis

Fourier transform infrared spectroscopy (FTIR) of the isolated compound was performed with a Thermo Nicolet model 6700 IR source range from 500 to 4000 cm^{-1} to obtain an IR spectrum to analyze the functional group present in the compound. High-resolution mass spectroscopy (HRMS) was used to determine the molecular mass of the compound using Agilent 6530B,

Agilent mass Q-TOF LC/MS. The structure of the isolated pure compound was determined with the help of nuclear magnetic resonance (NMR) spectroscopy using a Bruker Avance II 400 spectrometer (US).

Anti-QS Potential of the Isolated Pure Compound

The compound isolated from the fungal extract (2,4-DBP, a known compound) was further investigated for its anti-QS and anti-biofilm activity against *P. aeruginosa* as described below. To compare, BAC, a well-known phyto-compound known for its ability to attenuate the virulence factors of *P. aeruginosa* by downregulating the transcription of QS-regulated genes, was used as positive control. Dimethyl sulfoxide (DMSO) was used as a negative control. *P. aeruginosa* was grown in LB broth to attain an OD$_{600}$ of 0.4. It was further incubated in the presence of 2,4-DBP or BAC for 18 hrs. To obtain a cell-free culture supernatant for different assays, bacterial cells were pelleted down by centrifugation at 10,000 rpm for 10 min.

Determination of Sub-MIC and Growth Curve Analysis

Microbroth dilution, using the Clinical and Laboratory Standards Institute (CLSI) standard method as described in Luo et al. (2016), was used to determine the MIC of 2,4-DBP and BAC against *P. aeruginosa*. Consequently, sub-MICs were selected to perform further experiments.

Effect of 2,4-DBP on the Production of Virulence Factors

Violacein production assay
A visual investigation of the ability of 2,4-DBP to attenuate the QS-regulated violacein pigment production in *C. violaceum* was performed using agar well diffusion assay as described in Rajkumari et al. (2018a). A quantitative estimation of the inhibition of violacein production by *C. violaceum* was performed when treated with 2,4-DBP and BAC at respective sub-MICs by spectrophotometric measurement at 585 nm of the supernatant (Rajkumari et al., 2018a).

Pyocyanin production assay
A quantitative chemical assay was used to measure the inhibition of pyocyanin pigment production. Briefly, 1 mL of cell-free culture supernatant of *P. aeruginosa* grown with 2,4-DBP and BAC at appropriate concentrations was extracted with an equal volume of chloroform. After extraction, the organic phase was extracted by 1 mL of 0.2 N HCl, and the amount of pyocyanin was estimated spectrophotometrically at 520 nm (Ganesh and Rai, 2016).

Proteolytic Activity Assay
Pseudomonas aeruginosa secretes several proteases that serve as key mediators to establish an acute infection.

Chitinase activity assay
Modified chitin azure assay was used to determine the inhibition in chitinase activity (Husain et al., 2013). Chitin azure (0.5 mg/mL) dissolved in sodium citrate buffer (0.1 M, pH 4.8)

was used a substrate. Concisely, 1 mL of the cell-free supernatant of *P. aeruginosa* was mixed with 0.5 mL of substrate solution, and the mixture was incubated for 7 days at 37°C in constant agitation (150 rpm). After removal of the insoluble substrate by centrifugation at 10,000 rpm, absorbance of the collected supernatant was recorded at 570 nm.

LasA staphylolytic assay

LasA staphylolytic activity, an ability to lyse heat killed cells of *Staphylococcus aureus*, was estimated as described by Kessler et al. (1993). Harvested pellet of *S. aureus* cells grown overnight was resuspended in 0.02 M Tris (pH 8.5) to obtain an OD_{600} of 0.8. 100 µL of cell-free supernatant of *P. aeruginosa* (obtained as described above) was mixed with 900 µL of *S. aureus* suspension. After an incubation of 1 h, the cell density was measured at 600 nm.

LasA protease assay

Proteolytic activity of *P. aeruginosa* was estimated as reported by Hentzer et al. (2002) with some modifications. Briefly, 500 µL of substrate solution and 0.3% azocasein [prepared in 50 mM Tris (pH 7.8)] were mixed with 100 µL of cell-free supernatant of *P. aeruginosa* for 30 min at 37°C. Finally, 0.5 mL of prechilled 10% trichloroacetic acid was added and incubated for 15 min at 4°C to precipitate the undigested substrate. The protease activity was recorded as absorbance at 400 nm of the clear supernatant obtained after centrifugation at 10,000 rpm.

LasB elastase assay

The elastolytic activity of the cell-free supernatant of *P. aeruginosa* was measured according to Ohman et al. (1980). In brief, 100 µL of culture supernatant of *P. aeruginosa* was added to 900 µL of elastin congo red buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg of elastin congo red. The reaction mixture was incubated at 37°C for 3 h. Finally, the elastolytic activity was recorded as absorbance at 495 nm of the clear supernatant obtained after centrifugation at 10,000 rpm for 10 min.

Motility Assay

The effect of sub-MIC concentrations of 2,4-DBP and BAC on the motility, i.e., swimming and swarming ability of *P. aeruginosa*, has been investigated as described in Mishra et al. (2018). Treatment with BAC acted as a positive control whereas the untreated sample acted as internal control and 2,4-DBP acted as treatment.

Hydrogen Cyanide (HCN) Production Assay

The production of HCN by *P. aeruginosa* as one of its virulent factors was assayed according to Reetha et al. (2014). King's B medium agar plates supplemented with glycine were prepared with and without test compounds. After streaking *P. aeruginosa* onto the plates, a filter paper saturated with 0.5% picric acid, fortified with 2% of Na_2CO_3, was placed on the roof of the lid of the petri dish. The plates were tightly sealed and incubated for 24 h at 37°C. Production of HCN caused a change of color from yellow to orange.

Effect of 2,4-DBP on Biofilm Formation and Associated Factors of *P. aeruginosa*

Microtiter plate biofilm assay

The inhibitory effect of 2,4-DBP on the biofilm formation by *P. aeruginosa* was investigated according to Luo et al. (2016). *P. aeruginosa* was grown in 96-well flat-bottomed microtiter plates in the presence and absence of 2,4-DBP and BAC for 24 h at 37°C. After incubation, the wells are washed with sterile phosphate-buffered saline (PBS) to remove unadhered cells. The biofilm was stained with 1% crystal violet for 5 min and again washed with sterile PBS to remove excess stain. The crystal violet stained biofilm was dissolved with 33% acetic acid and was quantified by absorbance at 595 nm.

Extraction and quantification of exopolysaccharides (EPS)

The secreted exopolysaccharide (EPS) was quantified as reported by Packiavathy et al. (2014). The cell-free culture supernatant of *P. aeruginosa* was precipitated by three volumes of chilled ethanol (100%). It was incubated for 24 h at 4°C. The precipitated EPS was pelleted by centrifugation (10000 rpm, 15 min) and dissolved in Milli-Q water. EPS was quantified using the phenol-sulfuric acid method, wherein 1 mL of 5% cold phenol and 5 mL of conc. H_2SO_4 were mixed with 1 mL of EPS suspension, which was quantified spectrophotometrically 490 nm.

Extraction and quantification of rhamnolipids

Rhamnolipid extraction and quantification were performed as reported by Luo et al. (2017). *P. aeruginosa* was grown with and without 2,4-DBP and BAC (as described above), and 1 mL of cell-free culture supernatant (obtained as described above) was extracted with twice the volume of ethyl acetate and dried. The dried extract was resuspended in 900 µL of orcinol solution (0.19% orcinol dissolved in 53% v/v sulfuric acid). The mixture was incubated for 30 min at 80°C and quantified at 421 nm spectrophotometrically (Luo et al., 2017).

Extraction and quantification of alginate

The alginate extraction and quantification from the cell-free culture supernatant of *P. aeruginosa* were performed as described by Rashmi et al. (2018). Concisely, 0.6 mL of boric acid–sulfuric acid (4:1) solution was mixed with 70 µL of cell-free supernatant and vigorously mixed on an ice bath for 10 s. About 20 µL of carbazole solution (0.2% carbazole dissolved in ethanol) was added to the previous mixture, followed by centrifugation (10,000 rpm, 10 min). This was followed by incubation at 55°C for 30 min. Alginate was measured by absorbance at 530 nm.

Cell-surface hydrophobicity (CSH) assay

The methodology for estimating cell-surface hydrophobicity was employed as earlier reported by Viszwapriya et al. (2016) with minor modifications. Briefly, 1 mL of *P. aeruginosa* culture was cultivated with and without 2,4-DBP and BAC and mixed with 1 mL of toluene with vigorous vortexing for 2 min. The aqueous phase was collected for bacterial cell density measurement at 600 nm. The CSH indicated by the ability of cells to adhere to the hydrophobic substrate (here, toluene) was calculated as CSH% = [1 - (OD_{600} after vortexing/OD_{600} before vortexing)] × 100.

Congo red agar biofilm formation assay

The Congo red agar method was performed as reported by Kuzu et al. (2012). Congo red dye (0.08%) was added to the agar medium containing comprised brain heart infusion broth (BHI-37 gm/L), agar (1%), and sucrose (0.5%). Media plated were prepared with and without 2,4-DBP and BAC. *P. aeruginosa* was streaked on the congo red plates and incubated for 24–48 h at 37°C. The presence of dry crystalline black colonies confirmed the exopolysaccharide (EPS) production.

Extracellular DNA (eDNA) quantification

The supernatant of *P. aeruginosa* was filter sterilized through a 0.22-μm membrane and treated with an equal amount of phenol/chloroform/isoamyl alcohol (25:24:1), and the mixture was vortexed for a few seconds. The eDNA in the supernatant (500 μL) was then precipitated by sodium acetate (200 μL) and ice-cold isopropanol (1.3 ml). The precipitated eDNA was pelleted by centrifuging it at $12,000 \times g$ for 15 min at 4°C. The pellet obtained was resuspended in 40 μL of TE buffer (1 mM EDTA and 10 mM Tris, pH 8.0). The eDNA suspension was treated with 10 μL proteinase K (10 μg/μL) followed by incubation at 37°C for 1 hr. With the help of NanoDrop Fluorospectrometer, the eDNA was quantified and electrophoresed in agarose gel (0.8% [w/v] agarose in TBE buffer) and visualized in the gel documentation system.

Microscopic analysis of biofilm

The biofilm formation on the abiotic surface was assayed as described by Rajkumari et al. (2018a). In a 24-well microtiter well plate containing Tryptic Soy Broth and coverslips (1 × 1 cm), 1/100th diluted overnight *P. aeruginosa* broth culture was grown for 24 h at 37°C. For light microscopic analysis, the coverslips were washed with sterile PBS to remove unadhered cells and stained with 0.4% crystal violet for 10 min. For fluorescence microscope analysis, the coverslips were stained with acridine orange (4 μg/mL) in the dark and then washed with PBS to remove excess stain. The coverslips were allowed to dry and visualized under respective microscopes.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

The total RNA was extracted from Pseudomonas aeruginosa cultures grown for 18 h in the presence of the test compounds. The cultures were grown for 18 hrs to allow maximum exposure of the test compounds to the bacterium. The RNA isolation and cDNA synthesis were performed as described in Mahesh et al. (2020). The primers that were used in qRT-PCR are listed in **Supplementary Table S2**. Data were analyzed by the $\Delta\Delta Ct$ method. Each qRT-PCR reaction was performed in triplicates, and the assays were repeated thrice. Data were normalized to the housekeeping gene *rpoD* expression.

In silico Studies

Three-dimensional structures of ligands were docked to three-dimensional structures of proteins to check their binding affinity. This was followed by molecular dynamic simulations to get an insight into the effect of this binding on the three-dimensional structure of the proteins and the stability of the complex.

Docking

Molecular docking of LasR and RhlR with 2,4-DBP (which is isolated from *D. eschscholtzii* crude extract) was performed to evaluate their interaction strengths in comparison to their cognate ligands and BAC, using the "induced fit docking" module of Schrodinger (Schrodinger Inc., United States). These molecules were retrieved from PubChem, with the IDs as given in **Supplementary Table S3**. Protein was prepared through "protein preparation wizard" of the Schrodinger docking suite 2018. Ligands were prepared using the "ligprep" module.

Molecular Dynamic Simulation

Protein and protein–ligand complexes were simulated by Gromacs 5.1.4 simulation package using the "gromos" force field (Abraham et al., 2015). All the complexes were placed into a cubic box of size 2 Å along with the SPCE water model as the solvent. The system was equilibrated well before final simulation of 20 ns with the time step of 10 ps.

In vitro Infection Studies

The A549 lung epithelial carcinoma cells were infected with *P. aeruginosa* PAO1 in the presence of the identified compounds to evaluate if they interfere with host-cell infection by the bacterium. Host cells were grown in DMEM containing 10% FBS (fetal bovine serum) and L-glutamine–penicillin–streptomycin (0.5%) solution at 37°C in 5% CO_2 condition.

Adhesion Assay

The extent of (host) cell adhesion was evaluated by the procedure described in Hawdon et al. (2010). Confluent A549 cells were incubated with *P. aeruginosa* with a multiplicity of infection (MOI) of 100 (resuspended with DMEM) in the presence or absence of BAC (120 μg/mL) or 2,4-DBP (80 μg/mL) and incubated at 37°C for 1 h to allow bacterial adhesion. Wells with bacteria but no test compound served as positive control while the uninfected wells served as negative control. Uninfected host cells with BAC or 2,4-DBP were also kept. The wells were washed thrice with sterile PBS to remove non-adhered bacteria, followed by trypsinization with least possible trypsin lysed with 70 μL of 0.1% Triton X-100 (Sigma) at room temperature. The lysed cells were collected, serially diluted, and plated onto the LB agar plate for counting colony-forming units (CFU).

Invasion Assay

To enumerate the extent of host-cell invasion by *P. aeruginosa*, A549 cell invasion assay was performed according to Hawdon et al. (2010). Confluent A549 cells were infected with *P. aeruginosa* (resuspended with DMEM) at MOI of 100, in the presence or absence of BAC (120 μg/mL) or 2,4-DBP (80 μg/mL). The plates were incubated at 37°C for 2 h to allow internalization of the bacterial cells. Wells were washed with sterile PBS and then incubated with fresh DMEM supplemented with gentamicin (200 μg/mL) for 1 h to kill extracellular bacteria. Following incubation, the cells were washed thrice with sterile

PBS, trypsinized, and lysed with 70 μL of Triton X-100 (0.1%). The suspension was serially diluted and plated onto LB agar for CFU count. Host cells with bacteria but no test compound served as a positive control, while uninfected cells served as negative control. Uninfected host cells with BAC or 2,4-DBP were also kept. All cocultures were performed in triplicates.

Live/Dead Cell Imaging by Acridine Orange (AO)/Ethidium Bromide (EB) Staining

A549 cells were grown to about 90% confluence in a 35-mm cell-culture dish and infected with *P. aeruginosa* at MOI of 100 in the presence or absence of 2,4-DBP (80 μg/mL) and BAC (120 μg/mL). After 24 hrs of incubation, the medium was discarded, followed by washing of the cells thrice with sterile PBS. Finally, 20 μL of the AO/EB mix (4 μg/mL) was used to stain the cells and viewed under a fluorescent microscope with a B-2A filter (Nikon Eclipse TS100, Japan). EB stains only dead cells, whose membranes are permeable, whereas AO stains all cells. Hence, dead cells fluoresce red-orange while live cells fluoresce green.

Synergistic Antimicrobial Studies of 2,4-DBP With Antibiotics

Pseudomonas aeruginosa PAO1 was screened against therapeutically relevant antibiotics, which showed that the strain is resistant to ampicillin. Its MIC against the strain was determined by micro-broth dilution method as per CLSI guidelines. Following this, various combinations of ampicillin and 2,4-DBP (combinations) were used against *P. aeruginosa* to investigate any potential synergistic efficacy in inhibiting that bacterium. An overnight grown culture of *P. aeruginosa* was diluted to 1/100th, 200 μL of which was dispensed into the wells of 96-well microtiter plates and treated with the 2,4-DBP-ampicillin combinations. Untreated wells served as controls. Each treatment was performed as triplicates. After 24 h of incubation, the growth of the cells were monitored and expressed the cell viability in terms of percent CFU.

Mathematical Calculations and Statistical Analysis

Each experiment was performed in triplicates, and values were expressed as standard means with standard deviations. Values for 2,4-DBP-treated experiments are normalized with those BAC-treated whenever appropriate. All the cultures were adjusted to a set OD of 0.4 at 600 nm before the experiments.

The percentage of inhibition in different assays was calculated as follows:

$$\text{Percentage inhibition} = \left(1 - \frac{\text{Ab. (sample)}}{\text{Ab. (control)}}\right) * 100$$

where Ab. (control) = Absorbance of control, Ab. (sample) = Absorbance of treated sample, at respective wavelengths.

Statistical analyses of all the experiments were performed in Microsoft Excel MegaStat software. Data readings of all experiments were documented as mean ± standard deviation. The *p*-values < 0.05 represent the significance of the conclusion.

RESULTS AND DISCUSSION

Endophytic Fungus *Daldinia eschscholtzii* Shows Anti-QS Activity

A total of 32 endophytic fungi were isolated from the leaves of *Tridax procumbens*. Their extracts were screened against *P. aeruginosa* and *C. violaceum* for anti-QS activity. Out of them, the isolate with code TP2-6 showed the best activity and hence was selected for further studies (**Supplementary Table S1**).

A mature colony of TP2-6 was olivaceous green with smoky gray appearance at the surface texture. The margin was as follows: entire; colony fluffy in texture; surface color changes from white to dark gray and covers entire plate (90 cm) after 6 days of inoculation. The culture produced spores, which imparts a grainy appearance to the surface. Light microscopy revealed the hyphae to be septate, hyaline, to melanized thick walled as the colony ages. The conidia produced were small, numerous, hyaline, and ellipsoid with an attenuated base (**Figure 1A**). Apart from the morphological observations, the sequencing data confirmed it as *Daldinia eschscholtzii*. The sequence obtained in molecular analyses was submitted to GenBank with accession number KX987249.

Phylogenetic analysis of the sequence data consisted of Bayesian and maximum likelihood analysis as combined aligned dataset. The ITS dataset comprised 22 taxa and 647 characters from *Daldinia* species with *Hypoxylon fragiforme* as an outgroup. RAxML analysis of the ITS dataset yielded a best-scoring tree with a final maximum likelihood of 2060.50. In the maximum parsimonious dataset, of 647 total characters, 58 variable characters are parsimony-uninformative, and the number of parsimony-informative characters is 112. The parsimony analysis resulted in 10 equally parsimonious trees with a length of 253 steps (CI = 0.798, RI = 0.891, RC = 0.712, HI = 0.202). Bootstrap values of ML and MP equal to or above 75% based on 1000 replicates were shown (**Figure 1B**). Trees generated under maximum likelihood (ML), maximum parsimony (MP), and Bayesian analyses were similar in topology. The phylogenetic analyses show that our taxon groups together with *D. eschscholtzii* share a sister relation with *D. placentiformis*, *Daldinia caldariorum*, and *Daldinia albofibrosa* (99% ML/100 MP/1BYPP).

2,4-Di-Tert-Butylphenol Was Identified as the Bioactive Anti-QS Compound

Further, we were interested in isolating the bioactive compound from a crude extract of *D. eschscholtzii*. The crude extract from the *D. eschscholtzii* culture was prepared and subjected to column chromatography with increasing polarity from hexane to water with different ratios of solvents. Nine fractions were collected separately, which were subjected to bioactivity-guided fractionation. Active fractions were subjected to column chromatography with hexane: ethyl acetate (7:3) ratio, and fractions were collected in 20-mL volume aliquots. A single band was observed on the TLC plate from a purified fraction, and the purity was confirmed from analytical HPLC using the C_{18} column, using photodiode array

FIGURE 1 | Identification and molecular phylogenetic analysis of *Daldinia eschscholtzii*. **(A)** Microscopic observations for the identification of *D. eschscholtzii*. The colony morphology, mycelia, and spores stained in lactophenol cotton blue are shown. **(B)** Phylogram based on the RAxML analysis of the ITS-1 DNA sequence dataset. Bootstrap support values for ML, MP higher than 75%, and BYPP values greater than 0.90 are given above each branch respectively for *D. eschscholtzii* (TP2-6, an in-house code given to the isolate). **(C)** Molecular structure of 2,4-di-tert-butylphenol (2,4-DBP) identified as the bioactive compound that was isolated from *D. eschscholtzii*.

detectors (PDA) with model number SPD-M20A (Shimadzu, Japan) (**Supplementary Figure S1**). HPLC analysis of the purified sample revealed a major component of ≥95% and a few minor impurities. The purified fraction was subjected to high-resolution mass spectrometry (HRMS) to estimate the molecular weight of the compound (**Supplementary Figure S2A**). The molecular weight was established by Q-TOF (quadruple-time-of-flight) HRMS mass spectrometry, with mass of [M + H] + at m/z 207.17, corresponding to the major peak, which gives the accurate mass of ≈206.17, in the positive ion mode (**Supplementary Figure S2A**). FTIR spectra of a purified sample displayed a peak at 3511 cm^{-1}, which indicates stretching of the O–H phenolic group. Further, C–C stretch of the alkyl group was represented by the peaks at 2863–2951 cm^{-1}. Moreover, the peak observed at 1247 cm^{-1} reveals a C–O stretching of phenols. An aromatic C–C stretch was recognized by peaks at 1504–1604 cm^{-1} (**Supplementary Figure S2B**). Thus, evidences provided by

the above functional groups confirmed the phenolic nature of the compound.

^{1}H NMR data of the purified sample shows the occurrence of two singlets at 1.325 ppm and 1.447 ppm which resembles a di-substituted tertiary butyl group (**Supplementary Figure S3**). Another singlet around 4.662 indicates the presence of a phenolic hydrogen. The rest of the 3 protons were detected in the aromatic downfield region in between 6.602 and 7.332 ppm, which suggests it to be a tri-substituted aromatic compound. This data was further supported by ^{13}C NMR. The ^{13}C NMR spectra of the isolated pure compound exhibited the occurrence of 10 carbon signals, of which 6 were downfield carbon signals present in between 116.07 and 151.89 ppm which were in the aromatic region. The remaining four carbon signals were detected in the upfield region in between 29.81 and 34.87 ppm. Out of six carbons, the aromatic substitution was confirmed by the presence of three quaternary carbons at 135.32 ppm, 143.12 ppm, and 151.89 ppm. Moreover, the presence of a phenolic OH

group at quaternary carbon present at the most downfield region, i.e., 151.89 ppm, was confirmed in the molecule. Among the four carbon signals present in upfield regions, two were tertiary carbons at 34.87 ppm and 34.42 ppm while the rest of the two were methyl signals at 331.77 ppm and 29.81 ppm. Thus, the spectrum shows that in the compound, two tert-butyl substitutions are present on the remaining quaternary carbons at 135.32 ppm and 143.12 ppm in the aromatic ring. Therefore, after the interpretation of the hydrogen and carbon spectra, the structure of the compound was structurally elucidated as 2,4-di-tertbutylphenol ($C_{14}H_{22}O$) (**Figure 1C**). The final yield of 2,4-DBP ($\geq$95% purity) was 1.9 mg/L.

2,4-DBP Shows Anti-QS Activity Against *C. violaceum* and *P. aeruginosa*

The expression for the production of the violacein pigment by *C. violaceum* is regulated by QS. Therefore, any inhibitor of *C. violaceum* can be visually determined by the inhibition of production of the pigment. Hence, it has been used as a marker trait in QS studies as a reporter model (Stauff and Bassler, 2011). In our study, 2,4-DBP and BAC (positive control) were able to inhibit violacein production in the reporter strain on the agar plate (**Figure 2A**). The zone of inhibition of violacein production was 16 mm diameter for 2,4-DBP (80 μg/mL) and 18 mm for BAC (120 μg/mL). When expressed quantitatively, inhibition of violacein production when treated with 2,4-DBP (76% at 80 μg/mL) was comparable to that when treated with BAC (88% at 120 μg/mL) **Figure 2B**.

In earlier studies, inhibition zones of 10 mm and 13 mm of violacein production were reported for two phenethylamide metabolites isolated from marine *Halobacillus salinus* bacteria (Teasdale et al., 2009). In a more recent study, Noumi et al. (2018) reported 69.3% of violacein inhibition by tea tree oil. Our study achieved a stronger inhibition of violacein production by 2,4-DBP that we isolated from *D. eschscholtzii*. Since the molecule showed anti-QS activities, we hypothesized that this compound can inhibit growth of the notorious human nosocomial pathogen *P. aeruginosa*, or its virulence factors that are controlled by QS.

2,4-DBP Does Not Inhibit Growth of *P. aeruginosa*

The MIC based on the microbroth dilution method was calculated for both 2,4-DBP and BAC to examine if 2,4-DBP shows any inhibitory effect on the growth of *P. aeruginosa*. The MIC for 2,4-DBP was found to be >1024 μg/mL (not shown here). The growth pattern and change in the cell density of *P. aeruginosa* also remained largely unaffected when treated with three different concentrations: 40, 60, and 80 μg/mL of 2,4-DBP. This suggests that 2,4-DBP has no effect toward the growth kinetics of the bacterium, when compared to the growth in the presence of the sub-MIC level of BAC (120 μg/mL) (**Figure 2B**). This result was well in agreement with a similar observation that was reported by Viszwapriya et al. (2016) where 2,4-DBP showed a non-bactericidal effect on the growth of *Streptococcus pyogenes*.

Though the compound showed no effect toward the growth of the pathogen, inhibition of virulence factors of *P. aeruginosa*

that are controlled by QS could be an invaluable potential of 2,4-DBP, especially when it showed strong anti-QS activities. Therefore, we intended to study the effect of 2,4-DBP on the expression of QS-regulated genes and QS-regulated production of extracellular virulence factors, production of biofilm and its associated factors, and *in vitro* host-cell adhesion and invasion. The same concentrations of 2,4-DBP (40, 60, and 80 μg/mL) and BAC (120 μg/mL) were used throughout the rest of the study.

2,4-DBP Treatment Greatly Reduced Pyocyanin Production in *P. aeruginosa*

Pyocyanin, a predominant green-colored phenazine pigment and a redox-active toxin secreted by *P. aeruginosa*, critically plays a detrimental role for the establishment of an infection. The Rhl component of the QS system in *P. aeruginosa* activates the expression of pyocyanin production in conjunction with RhlR and the autoinducer signal molecule C4-HSL (Bratu et al., 2006). Pyocyanin also induces pathogen-driven neutrophil apoptosis by reducing local inflammation and creates a biofilm formation environment (Allen et al., 2005). In our study, 2,4-DBP treatment reduced the level of pyocyanin production by 60% whereas BAC reduced it by 69% without significantly affecting the bacterial growth (**Figure 2B**). This can also be visualized by the abrupt decrease in green color pigment in the supernatant of treated cultures as compared to those untreated (**Figure 2A**). This reduction was superior to those achieved by one previous study, wherein ethanolic extract, ethyl acetate extract, and N-butanol extract of *Camellia nitidissima* Chi flower at a concentration of 0.75 mg/mL reduced pyocyanin production by 51.2%, 56.9%, and 51.5%, respectively (Yang et al., 2018).

2,4-DBP Treatment Considerably Reduced Chitinase Activity *P. aeruginosa*

Chitinolytic activity by bacteria plays a significant role in chitin degradation, which results in recycling of a carbon as well as nitrogen source into a simply accessible form in the ecosystem (Gooday, 1990). The expression of chitinase enzyme enhances in clinical isolates, thus playing a role in virulent infection (Salunkhe et al., 2005). In our study, we recorded 2,4-DBP and BAC at concentrations of 80 and 120 μg/mL, respectively, to reduce chitinase activity in the culture extracts of *P. aeruginosa* (**Figure 2C**). The reduction in chitinase activity was found to be 27.1% and 30.8% in the case of 2,4-DBP and BAC, respectively, as compared to the untreated control. Interestingly, however, Husain et al. (2013) reported 80% inhibition in chitin production when *P. aeruginosa* was treated with 1.6% of clove oil.

2,4-DBP Causes Dose-Dependent Decrease in *P. aeruginosa* Protease Activity

Initial establishment of infection in host tissues is instigated by elastases and proteases. *P. aeruginosa* secretes several protease and elastase virulence factors regulated by LasIR, which implies their roles in its pathogenicity (Kessler et al., 1993). Elastase, a powerful T2SS-secreted proteolytic enzyme, is encoded by gene lasB. It has a wide range of substrates, including elements
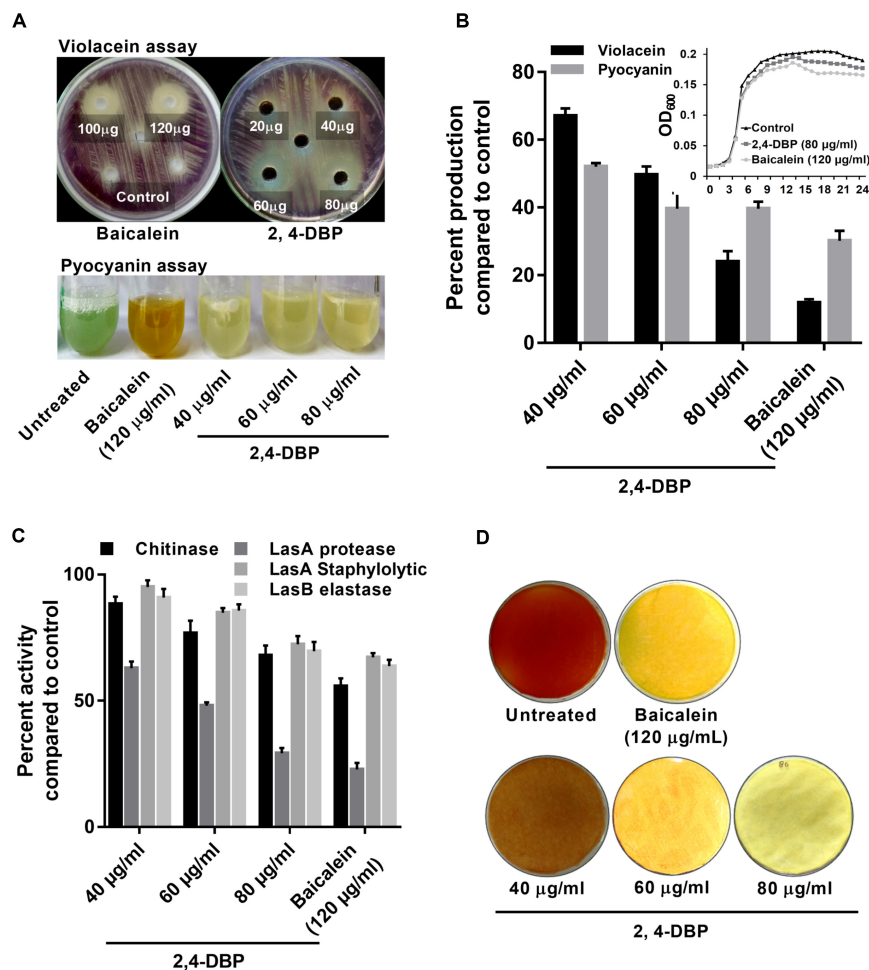
FIGURE 2 | Inhibition of virulence factors under control of quorum sensing by 2,4-di-tertbutylphenol (2,4-DBP). **(A)** Visual qualitative assay for inhibition of *Chromobacterium violaceum* violacein production and *Pseudomonas aeruginosa* pyocyanin production by 2,4-DBP at indicated concentrations. **(B)** Quantitative estimation of violacein and pyocyanin production. The growth curve in the presence of 2,4-DBP, baicalein, and untreated control is shown in the inlet. **(C)** Inhibition of chitinase, LasA protease, LasA staphylolytic activity, and LasB elastase activities by 2,4-DBP at indicated concentrations. **(D)** Visual qualitative assay of inhibition of *P. aeruginosa* HCN production by 2,4-DBP at indicated concentrations. Bar diagrams represent mean percentage values of triplicates normalized with those obtained with untreated controls.

of connective tissue such as elastin, collagen, fibronectin, and laminen. These bacterial proteases act as hydrolytic enzymes that target the host's proteins to facilitate the invasion and growth of the pathogen (Musthafa et al., 2011). In *P. aeruginosa*, expression of exoproteins such as alkaline protease and elastase is under the regulation of QS (Swift et al., 1996). In light of the ability of 2,4-DBP as a QS inhibitor, we investigated the role of 2,4-DBP on azocasein-degrading protease activity, LasA staphylolytic activity, and LasB elastase activity and observed a dose-dependent decrease in the protease activity of *P. aeruginosa* (**Figure 2C**). 2,4-DBP reduced LasA staphylolytic activity by 27.6% at 80 µg/mL and BAC by 32.8%. Equivalently, LasB elastase activity was attenuated by 30% as compared to BAC by 36.3%. Furthermore, LasA protease activity was found to get attenuated by 70.7% due to 2,4-DBP at 80 µg/mL whereas BAC reduced this to 77.1%. Although Rajkumari et al. (2018b) reported the decrease in staphylolytic activity by 21.8% and LasA protease by 71% when

treated with cinnamic acid, in the present study, 2,4-DBP exerted moderate effects on LasA staphylolytic activity and LasB elastase attenuation but significant reduction in LasA protease activity.

2,4-DBP Inhibits *P. aeruginosa* HCN Production

Production of HCN provides an advantage to *P. aeruginosa* to inhabit a range of ecological niches and hence contribute to its pathogenicity (Williams et al., 2006). Cyanide promptly diffuses in tissue and inhibits aerobic chain reaction by irreversibly binding to the terminal oxidases of respiratory chains and hence its profound toxicity (Zlosnik et al., 2006). In our study, treatment with 2,4-DBP resulted in attenuation of HCN production in contrast to untreated control. The HCN thus produced reacts with picric acid (yellow in color) in the presence of sodium carbonate, resulting in a color change from yellow to orange to

brick red. A sharp decrease in color change of filter paper from yellow to orange depicted less HCN production in case of 2,4-DBP and BAC at 80 and 120 µg/mL, respectively, whereas a deep-orange color was observed in drug-free control depicting high HCN production (**Figure 2D**).

2,4-DBP Strongly Inhibits Motility of *P. aeruginosa*

Pseudomonas aeruginosa possesses an exquisite mechanism to ingeniously use different types of motilities to facilitate colonization in several ecological niches. Motility of *P. aeruginosa* also plays a significant role in the surface attachment and maturation of biofilms (O'toole and Kolter, 1998). *P. aeruginosa* possesses a polar flagellum that aids in swimming and swarming on liquid and semisolid surfaces, respectively (Murphy, 2009; Murray et al., 2010). Herein, we investigated the swimming and swarming ability of *P. aeruginosa* when treated with 2,4-DBP and BAC. As evident from **Figures 3B,C**, the inhibition of swarming was 78% when treated with 2,4-DBP at 80 µg/mL, while the positive control BAC inhibited swarming by 73.9% at 120 µg/mL. Similarly, when treated with 2,4-DBP at 80 µg/mL, swimming was inhibited by 60.2%, while BAC reduced this to 51.5% (**Figures 3A,C**), suggesting that 2,4-DBP shows higher inhibitory effects on motility of *P. aeruginosa* than BAC does. According to earlier studies, the inhibitory effect of aspirin on the swimming motility of *P. aeruginosa* was 34% (El-Mowafy et al., 2014). Similarly, Li et al. in 2018 reported that cinnamaldehyde could restrict swarming up to 58.4% and swimming up to 40.7% at the concentration of 1 µL/mL. Compared to these earlier results, we could achieve greater inhibition of swimming as well as swarming motility of *P. aeruginosa* by 2,4-DBP.

2,4-DBP Attenuates *P. aeruginosa* eDNA Production

Extracellular DNA (eDNA) is a major constituent of the biofilm matrix of *P. aeruginosa*. eDNA is supposed to be produced from random chromosomal DNA from dead bacteria gibe strength to the biofilm matrix (Allesen-Holm et al., 2006). During starvation, eDNA acts as a nutrient source of *P. aeruginosa* (Finkel and Kolter, 2001). Extracellular DNA, furthermore, is known to ease the biofilm expansion mediated by twitching motility as it maintains organized cell arrangements to synchronize the movement of cells (Gloag et al., 2013). In this study, we observed a significant decrease in *P. aeruginosa* eDNA production when treated with 2,4-DBP (**Figure 4C**). The reduction of eDNA was recorded to be ∼82%, which is on par with the BAC, as quantified with ImageJ software.

2,4-DBP Significantly Impairs *P. aeruginosa* Biofilm Formation

Biofilms are contemplated as a 3D network of microbial communities adhering to biotic or abiotic surfaces, enveloped by an extracellular matrix comprised of bacterium-derived DNA, exopolysaccharides, and proteins released by the bacteria embedded therein (Chen et al., 2018). Biofilms are clinically one

of the most relevant features expressed by bacteria, since they act as an impermeable barrier to antibiotics and the host immune system, thus promoting antibiotic tolerance and persistence. The best known activator signal for biofilm formation is the QS signaling system. Hence, we investigated and quantified the deleterious effect of 2,4-DBP on biofilm formation by this bacterium. Notably, when compared to drug-free control, 2,4-DBP 80 µg/mL attenuated biofilm formation by 49%, marginally less (53%) than when treated with the positive control BAC at 120 µg/mL (**Figure 4A**). In the treatment, in contrast to 53% attenuation by BAC, biofilm attenuation by 2,4-DBP at a sub-MIC of 80 µg/mL was 49%, little less than the positive control. Disruption of the biofilm could also be directly observed under light microscopy when treated with 2,4-DBP. This can also be visualized on Congo red agar (CRA), which serves as a qualitative detection method for biofilm-positive bacteria. Bacteria that produce biofilms grow into dry crystalline black colonies when inoculated on a Congo red agar medium; they remain pink otherwise (Freeman et al., 1989). We observed the absence of black colonies of *P. aeruginosa* on Congo red agar when treated with 2,4-DBP, which is suggestive of the inability of the bacterium to produce a robust biofilm in the presence of test compounds (**Figure 4B**). A similar observation of biofilm with the help of light as well as fluorescent microscopy in **Figure 4D** corroborates with the fact that treatment with 2,4-DBP impairs the growth of biofilm development in the initial phase itself and inhibits biofilm development as compared to control.

In earlier reports, reduction in biofilm by phenolic compounds such as cinnamic acid, ferulic acid, and vanillic acid was 44, 45, and 46%, respectively (Ugurlu et al., 2016), suggesting 2,4-DBP as a potential anti-biofilm candidate. Similarly, Padmavathi et al. (2015) evaluated anti-fungal and anti-biofilm efficacy of 2,4-DBP and observed a strong anti-fungal action by inhibiting and disrupting biofilm formation in *Candida albicans*. Our results are also supported by reports of 2,4-DBP displaying a concentration-dependent biofilm inhibition that can reach a maximum of 79% 48 µg/mL concentration (Viszwapriya et al., 2016) in *Streptococcus pyogenes*. In addition, in a more recent study, Rajkumari et al. (2018b) described that the treatment with betulin and betulinic acid possessing an anti-QS ability resulted in the formation of pink colonies of *P. aeruginosa* on Congo red agar and hence reduced biofilm formation.

2,4-DBP Significantly Inhibits Production of Exo-Polysaccharides

Extracellular polymeric substances (EPS) are the main constituent of *P. aeruginosa* biofilms and crucial for its biofilm architecture (Kreft and Wimpenny, 2001). EPS helps *P. aeruginosa* to evade antibiotic treatment and immune responses (Ghafoor et al., 2011). Besides providing mechanical stability to biofilm through various interactions, EPS defend bacterial cells by impeding penetration and/or sequestering of antimicrobial agents (Donlan, 2002; Ryder et al., 2007). In our study, we found that, when bacterial cells were exposed to 2,4-DBP, the production of EPS was reduced by 34.4%, while the reduction was 36% for BAC (**Figure 4A**). This marked

FIGURE 3 | Inhibition of *Pseudomonas aeruginosa* swimming motility **(A)** and swarming motility **(B)** by 2,4-di-tert-butylphenol (2,4-DBP) at the indicated concentrations. **(C)** Quantitative estimations of the inhibition of *P. aeruginosa* swimming and swarming motility by 2,4-DBP. Bar values indicate mean percentages of triplicates normalized with those obtained with untreated controls.

FIGURE 4 | Inhibition of *P. aeruginosa* biofilm formation and biofilm-associated factors by 2,4-di-tertbutylphenol (2,4-DBP) at the indicated concentrations. **(A)** Inhibition of *P. aeruginosa* biofilm formation, production of alginate, exo-polysaccharide (EPS), and rhamnolipid, and hydrophobicity. Bar values indicate mean percentages of triplicates normalized with those obtained with untreated controls. **(B)** Congo red agar visual qualitative assay for inhibition of biofilm formation. **(C)** Inhibition of eDNA production by baicalein and 2,4-DBP. The bar values show intensity of the bands as estimated by ImageJ software, from the agarose gel electrophoresis image shown below. **(D)** Fluorescence and light microscopic images of biofilms when treated with various concentrations of baicalein and 2,4-DBP. Two random representative images are shown for each treatment. The scale bars are equivalent to 25 μm.

decrease on par with the positive control reflects the ability of 2,4-DBP as a potential anti-biofilm candidate. Similar reports were also present wherein the decrease in EPS was 31.2% by botulin, 18% by betulinic acid, and 31% by clove oil (Husain et al., 2013; Rajkumari et al., 2018b). In addition, 2,4-DBP inhibited EPS production by *Candida albicans* by 33%, (Padmavathi et al., 2015) and up to 33–46% in *Streptococcus* sp. (Viszwapriya et al., 2016), which are similar to that of our results.

2,4-DBP Causes Significant Reduction of *P. aeruginosa* Rhamnolipid Production

Rhamnolipid is an extracellular virulent factor and a prerequisite for biofilm establishment. It actively maintains *P. aeruginosa* biofilm architecture and reduces adhesion between bacterial cells (do Valle Gomes and Nitschke, 2012). In their involvement in early cell-to-surface interactions, further maintenance following dispersion/disruption of the biofilm is indispensable (Davey et al., 2003). Rhamnolipid is a biosurfactant composed of a rhamnose-containing glycolipid detergent-like structure and is well known to solubilize the phospholipids of lung surfactant, hence more prone to cleavage by phospholipase C (Köhler et al., 2010). We witnessed a reduction in rhamnolipid production when exposed to 2,4-DBP by 29.2%, compared to 38.8% for BAC (**Figure 4A**). Our study achieved better reduction in rhamnolipid production when compared to previous reports, wherein 19.03% and 21.61% reductions in rhamnolipid production were achieved when treated with betulin and betulinic acid at 125 μg/mL concentration (Rajkumari et al., 2018b).

2,4-DBP Impedes *P. aeruginosa* Alginate Secretion

Pseudomonas aeruginosa secretes alginate, a major polysaccharide component of the *P. aeruginosa* EPS component, which determines its surface characteristics such as hydrophobicity, charge, and electrostatic interactions of the cell surface with the surface (Herzberg et al., 2009). Alginates shield bacteria from adverse conditions and enhance surface adhesion (Boyd and Chakrabarty, 1995). Its production by *P. aeruginosa* aids in antibiotic resistance, phagocytic evasion, resistance toward macrophages and neutrophils, and scavenging of reactive oxygen intermediates (Cody et al., 2009). In our study, 2,4-DBP at 80 μg/mL impeded *P. aeruginosa* alginate secretion by 50%, while BAC treatment at 120 μg/mL resulted in a reduction by 55% (**Figure 4A**). This finding is significant as it corroborates with the previous reports of cinnamon oil, reducing alginate production by 54% at 0.2 μl/mL (Kalia et al., 2015).

2,4-DBP Significantly Decreases *P. aeruginosa* Cell-Surface Hydrophobicity

Hydrophobicity on bacterial surfaces plays a determinant role in the adhesion and biofilm formation of bacterial pathogens on animate as well as inanimate surfaces (Rosenberg and Doyle, 1990). The ability of *P. aeruginosa* to adhere to hydrocarbons is a measure of cell-surface hydrophobicity. A greater CSH is suggestive of a greater ability of the bacterium to adhere. This is achieved by shielding the repelling forces amid the surface charges, which is critically needed for early micro-colony formation during biofilm development (Pamp and Tolker-Nielsen, 2007). Hence, CSH is regarded as a major determinant of biofilm formation (Silva-Dias et al., 2015). In this study, CSH was reduced by 51.2% and 49.2% when treated with 2,4-DBP and BAC, respectively, which suggest its role in inhibiting adhesion of *P. aeruginosa* (**Figure 4A**), which is suggestive of a reduction in biofilm formation of the pathogen, as noted in the above results. In a similar study, 2,4-DBP resulted in significant reduction up to 70% in cell-surface hydrophobicity of *Streptococcus* sp.

FIGURE 5 | Relative expression of *P. aeruginosa* PAO1 *lasA*, *lasI*, *rhlR*, and *rhlI* genes under control conditions (DMSO) and following addition of 2,4-di-tert-butylphenol (2,4-DBP) (80 μg/mL). *indicates that $p < 0.05$ and hence statistically significant which was assessed by two tailed *t*-test.
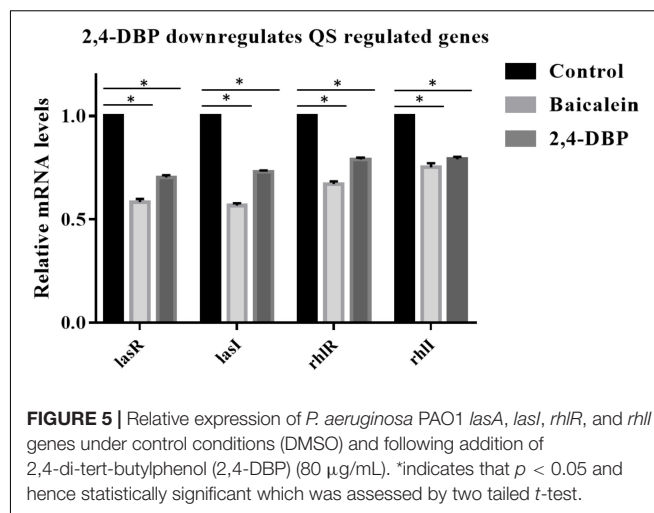
Since 2,4-DBP inhibited cell-surface hydrophobicity of *P. aeruginosa* considerably, we hypothesized that the test compound could also inhibit adhesion of the pathogen to its host cells.

2,4-DBP Downregulates QS Genes of *P. aeruginosa*

Since 2,4-DBP strongly inhibits the QS and secretion of virulence factors of *P. aeruginosa*, we hypothesized that 2,4-DBP might inhibit the expression of QS-related genes. To investigate this, we quantified the expression levels of QS-associated genes such as *lasI*, *lasR*, *rhlR*, and *rhlI* in *P. aeruginosa* which are treated with 2,4-DBP by using quantitative RT-PCR. We found that the treatment of *P. aeruginosa* with 2,4-DBP at 80 μg/ml concentration decreased the mRNA level of all the four QS-associated genes *lasI*, *lasR*, *rhlR*, and *rhlI RhlR* significantly on par with the positive control BAC, a well-studied inhibitor of QS (**Figure 5**). These results suggest that 2,4-DBP inhibits QS by downregulating the expression of QS-related genes.

2,4-DBP Causes Strong Inhibition of *P. aeruginosa* Host-Cell Adhesion

Host-cell adhesion of *P. aeruginosa* is the initial and decisive stage of colonization in the host and is crucial for an infection by the bacterium to be established. To evaluate whether 2,4-DBP impairs host-cell adhesion of *P. aeruginosa*, we infected A549 human alveolar carcinoma cells with *P. aeruginosa* in the presence of 2,4-DBP and BAC. The adhered cells were harvested and enumerated by CFU counts. We recorded an abrupt reduction in *P. aeruginosa* host-cell adhesion by 72% in the presence of 2,4-DBP at 80 μg/mL, as compared to 62% reduction in the presence of the positive control BAC at 120 μg/mL (**Figure 6**). The reduction in host-cell adhesion achieved in our study was remarkably higher than those achieved in previous studies, wherein it was 26.3% by the antibiotic ciprofloxacin at a concentration of 0.063 μg/mL, 16.4% by dextran at 5 mg/mL, 45.2% by an extract of soybean at 4.3 mg/mL, and 54.5% by a cranberry extract at 2.6 mg/mL (Ahmed et al., 2014).

This makes 2,4-DBP an attractive candidate for anti-virulence therapeutic strategies, whereby the pathogen can be sensitized to antimicrobials and/or the host's immune system, especially when biofilm-related infection is widespread and multidrug resistance in *P. aeruginosa* is rampant.

2,4-DBP Causes Severe Impairment of *P. aeruginosa* Host-Cell Invasion

Pseudomonas aeruginosa is known to escape the host's immune system by promoting its own internalization into host non-phagocytic host cells (Chi et al., 1991; Engel and Eran, 2011). To evaluate if 2,4-DBP is able to impair *P. aeruginosa* host-cell invasion, we infected A549 cells with *P. aeruginosa in vitro* at a multiplicity of infection of 100 in the presence of 2,4-DBP and BAC. The non-internalized bacteria were killed by gentamicin treatment; the internalized bacterial cells were harvested and enumerated by CFU counts. In the presence of 2,4-DBP at 80 μg/mL, host-cell invasion was severely reduced by 75%, whereas this reduction was 50% when infected in the presence of the positive control BAC at 120 μg/mL (**Figure 6**). Such a remarkable reduction in the host-cell invasion of bacterial cells depicts the potential of 2,4-DBP when compared to other studies, wherein invasion was decreased by about 45% when treated with ciprofloxacin at the concentration of 0.063 μg/mL, and 25% in the case of dextran at 5.0 mg/mL. However, a significant reduction was achieved when treated with an extract of soybean at 4.3 mg/mL, in combination with ciprofloxacin and dextran (Ahmed et al., 2014).

2,4-DBP Interferes With Host-Cell Death by *P. aeruginosa*

The ability of *P. aeruginosa* getting internalized eventually leads to induction of apoptosis, which is the tangible virulence of the bacterium resulting in host tissue damage. To investigate if our test compound can protect the host cells from the induction of apoptosis induced by the internalized bacteria, we infected the host cells with the pathogen at a multiplicity of infection of 100, followed by elimination of the extracellular bacteria. Host-cell death was observed by live dead cell imaging after 24 h of incubation. The cells were stained briefly with Acridine Orange/Ethidium Bromide solution and directly observed by fluorescence microscopy. Our results depict that bacterial cells treated with BAC resulted in more A549 cell deaths after 24 h of incubation while the degree of death induced in 2,4-DBP at the 80-μg/mL treatment was less (**Figure 6**).

Synergistic Studies of 2,4-DBP With Antibiotics

The MIC of ampicillin and 2,4-DBP was found to be more than 1024 μg. It means *P. aeruginosa* was resistant to the antibiotic and grows without any constraints even in the presence of 2,4-DBP at 80 μg/mL, as mentioned earlier. We were interested to investigate the combined effect of ampicillin and 2,4-DBP on *P. aeruginosa* and hence several combinations of ampicillin and 2,4-DBP were used in different concentration. As shown in **Figure 7**, the combination of ampicillin at 100 μg/mL and 2,4-DBP at 100 μg/mL was effective in eradicating the bacterial

growth as the bacterial cell viability at this combination was less than 2%. Furthermore, as evident from **Figure 7**, even the effect of concentration of ampicillin at 50, 75, and 100 μg/mL alone is similar and ineffective in killing *P. aeruginosa*. However, the introduction of 2,4-DBP even at the 40-μg/mL concentration in combination with ampicillin results in sharp deleterious effects on the bacterium. Hence, it could be presumed that 2,4-DBP was capable of weakening the bacterial cells, and further, ampicillin was able to kill the weakened pathogen, which earlier was ineffective at even higher concentrations. A similar sort of study was performed by Viszwapriya et al. (2016), where 2,4-DBP reduced the MIC of the standard antibiotic. A marked decrease in MIC value of erythromycin and tetracycline was observed in combination with 2,4-DBP against *Streptococcus* sp.

Docking Analysis of 2,4-DBP

The three-dimensional structure of LasR was retrieved from the PDB database. Bottomley et al. (2007) reported the crystal structure of the LasR receptor protein at 1.80 Å resolution. The NCBI CD database search of this protein revealed that it contains an autoinducer domain from residues 20 to 160, which is crucial for the transcription process (Marchler-Bauer et al., 2010). The complete structure details of this protein is discussed by Gopu et al. (2015). As the three-dimensional structure of RhlR is not solved, the predicted model, which was reported earlier (Rajkumari et al., 2018b), was used in this study. Molecular docking studies were performed to find out the hotspot residues of the protein.

Ligand molecule, 2,4-DBP, was docked in order to study the inhibition mechanisms. The signaling molecule showed less dock scores as compared to the ligand molecules (2,4-DBP and BAC). Information on all the interacting atoms of protein and ligands along with H-bond distances is provided in **Supplementary Table S4**. The pose of ligands in the complex with LasR and RhlR receptor proteins is shown in **Figures 8A,B**, respectively. This docking study revealed the hotspot residues of protein, which interacted with ligands. Few other residues were also noticed to interact with ligands and were highlighted in bold.

Molecular Dynamics Simulation

Molecular dynamics simulation studies were performed to study the conformational changes in proteins' three-dimensional structure for activation and deactivation of the LasR receptor protein in the presence of respective ligands. The simulations were performed with six complexes, LasR + signaling, LasR + BAC (LasR + BAC), LasR + 2,4-DBP, RhlR + 2,4-DBP, RhlR + BAC, and RhlR + 2,4-DBP. RMSD profiles of all simulated complexes were generated to study the protein deviation throughout the simulation period. The simulations were run for 20 ns with the time step of 10 ps and are shown in **Figure 9A**. The RMSD profile of this protein revealed that the protein-signaling molecule complex showed more deviation as compared to other two complexes. The same pattern of deviation was also revealed by three complexes of the RhlR protein. This instability in the three-dimensional conformation was caused because the crystal structure of the signaling molecule was crystallized with the signal molecule, which handles the
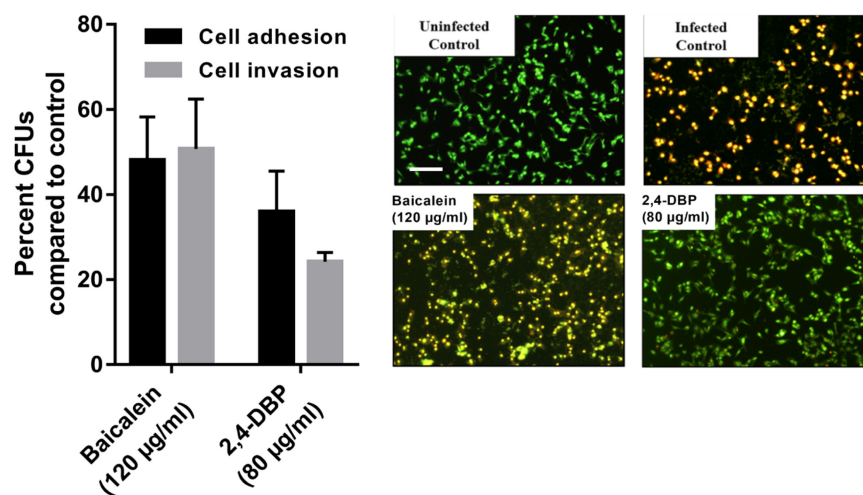
FIGURE 6 | Inhibition of *P. aeruginosa* host-cell adhesion and host-cell invasion by 2,4-di-tert-butylphenol (2,4-DBP) in untreated control, baicalein, and 2,4-DBP in the A549 lung epithelial cell infection model (left). Live/dead imaging of A549 lung epithelial cell by AO/EB, when infected with *P. aeruginosa* PAO1 in untreated control, when treated with baicalein (120 μg/mL) and 2,4-DBP (80 μg/mL) (right). The scale bar is equivalent to 20 μm.
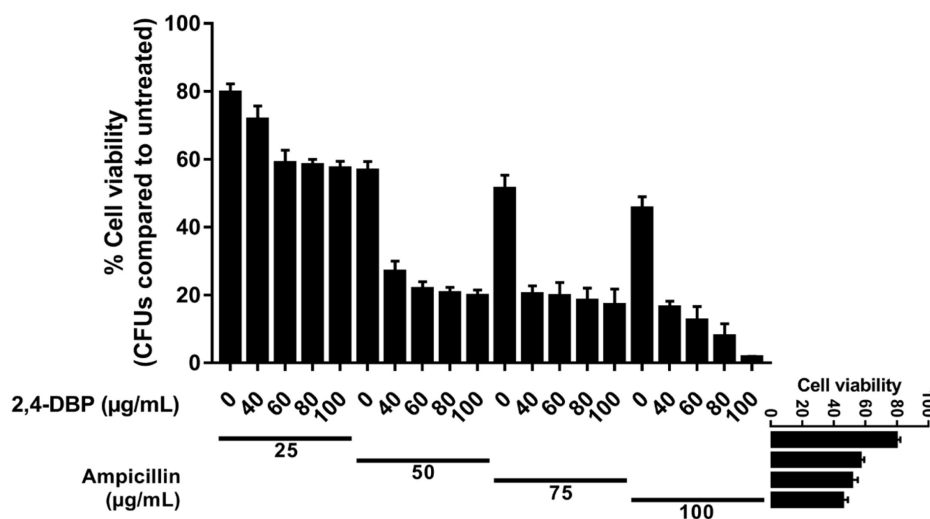
FIGURE 7 | Synergistic studies of 2,4-di-tert-butylphenol (2,4-DBP) with ampicillin: Graph showing cell viability of *P. aeruginosa* when treated with ampicillin and combination of ampicillin and 2,4-DBP.

activation of the LasR protein. The protein + BAC and ligand complexes showed the least deviation in comparison to the protein-signaling molecule.

Deviation in RMSD profile in a protein can occur for several possible reasons; it can be due to either expansion or contraction of the protein, or it may be because of folding of protein in the other direction. RMSD data was followed by analysis of radius of gyration (**Figure 9B**) to understand the deviation pattern of proteins. The expansion showed by the protein-signaling molecule was higher among all simulated complexes, whereas it was less in protein–ligand complexes. Both the proteins consist of the same cavity as an active site. The activation of the LasR protein depends on the availability of this cavity for the

signaling molecule. SASA (solvent-accessible surface area) graphs (**Figure 9C**) were generated to study how accessibility of this protein is affected due to opening or closing of the binding site. Keen observation of the SASA graph revealed that the protein had lost its surface accessibility when it interacts with 2,4-DBP.

In silico analysis provides insights into protein three-dimensional structures at the atomic level. The structural details of protein and ligands provide more details to improve the efficiency of drugs. A molecular docking study of the LasR receptor protein with both 2,4-DBP and signaling molecules revealed that they bind rigidly to the receptor. The structural comparison of both signaling molecules revealed that they shared a similar backbone. The functional groups of both molecules

FIGURE 8 | Docking of LasR **(A)** and RhlR **(B)** with their cognate ligands (3-oxo-C12-HSL and C4-HSL, respectively), baicalein and 2,4-di-tertbutylphenol (2,4-DBP). Three-dimensional structures of the LasR–cognate ligand complex and RhlR–cognate ligand complex are shown at the top of both the panels. For each complex, stick representations of the ligand molecule, along with its interacting protein residues, are shown on the right of each panel.

are different, and hence, their interaction pattern is different. The interaction of this functional group with protein residues may lead to these conformational changes. The RMSD profile of all the complexes showed that the protein-signaling complex of both proteins was the most dynamic complex whereas the protein + 2,4-DBP complex is the most rigid complex found in both the cases. The least deviation shown by the protein + 2,4-DBP complex indicates a stable complex and hence suggests this as a potential inhibitor of both the proteins. A similar pattern shown by the 2,4-DBP molecule as well as known inhibitor molecules proves that this ligand may be a potential inhibitor. The radius of gyration and SASA study revealed that this 2,4-DBP interacts with proteins strongly. The interactions of ligand had induced confirmation changes in the LasR receptor in such a way that the active site is no longer available to interact with other molecules. It was proved through molecular docking and molecular dynamics simulation studies that 2,4-DBP can act as an anti-quorum agent against *P. aeruginosa*.

SUMMARY AND CONCLUSION

To face the recurring breakdown of antibiotics success against *P. aeruginosa* infections, recent efforts have switched toward exploiting QS inhibitors as anti-pathogenic strategy. The prospective of this strategy is encouraging since the majority of such compounds are of natural origin produced by organisms.

More efforts are now being put in this concept, and it is acquiring momentum, being apparent from the extensive studies correlated with anti-QS subjects. In the present study also, 2,4-DBP, a proposed anti-QS compound, was isolated from *D. eschscholtzii*, a foliar endophytic fungus associated with *T. procumbens*. *D. eschscholtzii* in general is a wood-inhabiting/decaying endophytic fungi prevailing in warm tropical climate. On account of their endophytic habit, *Daldinia* sp. possess the trait of early colonization (Stadler et al., 2014). This fungus manifests a multitude of secondary metabolites as evident from fascinating data in the last decade (Ng et al., 2016). To this end, the isolated 2,4-DBP was assessed for its anti-QS activity and anti-biofilm activity against *P. aeruginosa*, a well-known opportunistic pathogen. We found that 2,4-DBP showed significant activity in inhibiting the production of various virulent factors such as pyocyanin, chitinase, several proteases, and biofilm-associated factors along with deleterious effects on biofilm formation. The Las and Rhl systems are closely related and are known to control the development of various virulence factors including alkaline protease, elastase, exotoxin A, lectins, pyocyanin, rhamnolipids, superoxide dismutase, and biofilm formation (Venturi, 2006). There is no direct evidence for swimming motility and alginate synthesis under the control of QS (Ledgham et al., 2003). However, along with the other virulence factors, these two phenotypes are often affected when QS is inactivated or inhibited (Bala et al., 2011; Gupta et al., 2016), hence suggesting that the regulatory modules responsible for
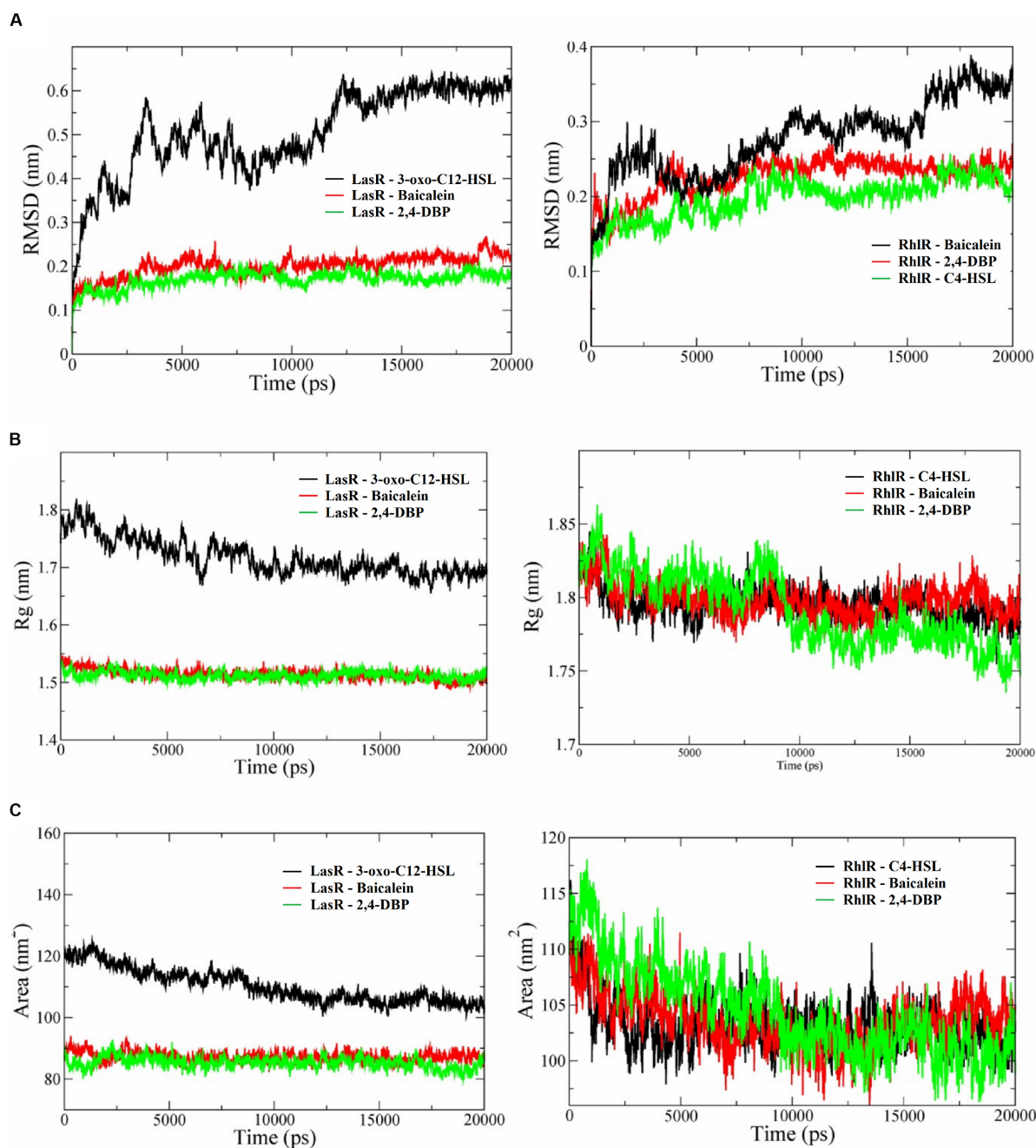
FIGURE 9 | (A) Root mean square deviation (RMSD) profiles **(A)**, radius of gyration graphs **(B)**, and SASA (solvent-accessible area) graphs **(C)** of *P. aeruginosa* LasR and RhlR receptor proteins complexed with their cognate ligands 3-oxo-C12-HSL and C4-HSL, respectively, baicalein and 2,4-di-tertbutylphenol (2,4-DBP). In all the graphs, the receptor protein is less stable than with baicalein and 2,4-DBP.

these two phenotypes may at least partially overlap with the QS signaling network (Luo et al., 2016).

In silico studies also showed its ability to bind to QS-regulated receptor proteins, Las and Rhl, and inhibit the binding of cognate signal molecules to inhibit QS. In earlier reports, 2,4-DBP is reported to curtail the killing of *Caenorhabditis elegans* up to 73%, when infected with *Streptococcus pyogenes*,

while *S. pyogenes* was capable of killing 100% *C. elegans* after 96 hrs of infection in control. Hence, this compound could play a role as a therapeutic agent (Viszwapriya et al., 2016). Moreover, in *S. pyogenes*, 2,4-DBP effectively reduced biofilm formation, EPS production, and cell-surface hydrophobicity and restricted the initial adhesion of bacterial cells during biofilm formation. For bio-monitoring eco-toxicological studies,

C. elegans is popularly used. As in Viszwapriya et al. (2016) who used *C. elegans* for assessing the toxicity of 2,4-DBP, they concluded the non-toxic nature of 2,4-DBP and considered it as apt for clinical applications. Apart from the present study, 2,4-DBP was found to be potent in constraining the biofilm formation besides considerably disrupting ($p < 0.05$) preformed biofilms in *C. albicans* (Padmavathi et al., 2015). In addition, 2,4-DBP, a known antioxidant, was investigated for its role in modulating the EPS in *Serratia marcescens* (Padmavathi et al., 2015). They reported a significant reduction in protein, polysaccharides, and eDNA components of EPS by *S. marcescens* when treated with 2,4-DBP, which would perhaps assist in biofilm disruption by facilitating the dissemination of antimicrobials into the biofilm. Recently in 2019, 2,4-DBP was reported to be isolated from *Bacillus licheniformis*, a thermophilic bacterium that thrives at 55°C the antibacterial activity against *S. aureus* and *P. aeruginosa* (Aissaoui et al., 2019). Hence, aforementioned studies support our results and provide evidence of 2,4-DBP as a potential candidate as a therapeutic agent. Besides the role of 2,4-DBP against bacteria and fungi, it is also reported to possess anticancer activity against human gastric adenocarcinoma AGS cells (Song et al., 2018).

Though LasR sits at the top of the *P. aeruginosa* QS hierarchy, *rhl* and *pqs* signaling regulons only partially overlap with *las* (Lee and Zhang, 2015). Therefore, inactivation or inhibition of the LasIR QS system may partially impair the Rhl and PQS signaling systems. There is considerable evidence that the Rhl signaling system negatively regulates the type-III secretion system (Hogardt et al., 2004; Bleves et al., 2005). Furthermore, previous reports also suggest that virulence of *P. aeruginosa* remains active in the $\Delta lasR$-$\Delta rhlR$ double mutant, possibly due to the secretion of the effectors ExoT and ExoS by the type-III secretion system (Soto-Aceves et al., 2019). In our study, we observed that, though the treatment of 2,4-DBP reduces the QS gene expressions by 30–40%, the organism was still non-virulent when cocultured with the host cells (**Figure 5**). This suggests that, though this reduction in QS genes is not huge, it is sufficient to reduce the production of the virulence factors by 50–70%, which is definitely profound, and to possibly keep the *rhl* system fairly active to inhibit the type-III secretion system, further inhibiting virulence. Additionally, 2,4-DBP offers a possibility of its use as combination therapy with antibiotics as obsolete as ampicillin against multidrug-resistant *P. aeruginosa*. While further studies are needed to validate this interesting dual property of 2,4-DBP, molecules with such properties can serve as valuable therapeutic options. In this respect, our study with 2,4-DBP could serve as a starting point for the identification of such molecules, which can cause all-round inhibition of virulence as well as help in killing the pathogen.

By only reducing QS using a quorum-sensing inhibitor, it may not be possible to treat *P. aeruginosa* infections. However, the best approach might be to attenuate quorum-sensing-mediated traits, such as virulence and biofilm formation, as well as to combine this with clinically relevant drugs that together with the host's immune system can act simultaneously to clear the pathogen. The main idea of our study was to achieve this by the use of 2,4-DBP. The considerable effect of the combination of 2,4-DBP with an obsolete antibiotic such as ampicillin achieves this to a large extent. It should be noted that it is not the inhibition of QS that makes 2,4-DBP valuable. Its ability of all-round inhibition of *P. aeruginosa* biofilm formation, production of virulence factors, and killing of the pathogen in combination with ampicillin, in addition to inhibiting QS, makes it a potential combination therapeutic agent.

Further, approaches to QS intervention claim to attenuate bacterial virulence without specifically inhibiting bacterial growth, suggesting that the immune system can regulate the infections *in vivo*. Nevertheless, strong experimental evidence against the validity of most of these hypotheses has emerged in recent years for the QS inhibitor in García-Contreras (2016). Moreover, many researchers believe that there are several challenges and limitations in anti-QS therapies that highlight three major properties attributed to QS inhibitors (Krzyżek, 2019). In order to develop truly solid QS inhibitor therapeutic alternatives to combat this remarkable pathogen, a much better understanding of its virulence and actions during infections is necessary. Even though the laboratory results are promising, it is undeniable that there is the need of thorough understanding of the knowledge of the impact of QS inhibition on the pathogen fitness in more convincing circumstances, such as interactions with a host, the external environment, and complex microbial communities (Liu et al., 2018).

To summarize, studies on anti-QS compounds/extracts from fungal sources are very few and are of recent origins. In fact, this has become a handicap for us to compare our results with other studies involving fungal extracts. Hence, we were forced to compare anti-QS activities of extracts/compounds of bacterial/plant origin. Nevertheless, it shows that the natural products are still the largest reservoir of compounds/metabolites for a range of ailments and for therapeutic use with fungi falling in line in the anti-QS realm also. To that extent, the present study revealed a promising role for selected fungi, isolated as endophytes from *T. procumbens*, after an initial screening. Also, we could isolate a pure compound 2,4-DBP from one of these fungi and demonstrate its potential as an anti-QS compound through various assays and experiments.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Genbank, KX987249.

AUTHOR CONTRIBUTIONS

RM and JK contributed to the conception, design, and experiment of the study. MK performed qRT-PCR and cell culture studies. CM performed the *in silico* studies under supervision of AM. RM wrote the first draft of the manuscript. SP, MK, CM, and AD wrote the sections of the manuscript. VV supervised the study.

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SUPPLEMENTARY MATERIAL

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Phyto-Mediated Synthesis of Porous Titanium Dioxide Nanoparticles From *Withania somnifera* Root Extract: Broad-Spectrum Attenuation of Biofilm and Cytotoxic Properties Against HepG2 Cell Lines

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There is grave necessity to counter the menace of drug-resistant biofilms of pathogens using nanomaterials. Moreover, we need to produce nanoparticles (NPs) using inexpensive clean biological approaches that demonstrate broad-spectrum inhibition of microbial biofilms and cytotoxicity against HepG2 cell lines. In the current research work, titanium dioxide (TiO_2) NPs were fabricated through an environmentally friendly green process using the root extract of *Withania somnifera* as the stabilizing and reducing agent to examine its antibiofilm and anticancer potential. Further, X-ray diffraction (XRD), Fourier transform infrared (FTIR), scanning electron microscopy (SEM), transmission electron micrograph (TEM), energy-dispersive X-ray spectroscopy (EDS), dynamic light scattering (DLS), thermogravimetric analysis (TGA), and Brunauer-Emmett-Teller (BET) techniques were used for determining the crystallinity, functional groups involved, shape, size, thermal behavior, surface area, and porosity measurement, respectively, of the synthesized TiO_2 NPs. Antimicrobial potential of the TiO_2 NPs was determined by evaluating the minimum inhibitory concentration (MIC) against *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, *Listeria monocytogenes*, *Serratia marcescens*, and *Candida albicans*. Furthermore, at levels below the MIC ($0.5 \times$ MIC), TiO_2 NPs demonstrated significant inhibition of biofilm formation (43–71%) and mature biofilms (24–64%) in all test pathogens. Cell death due to enhanced reactive oxygen species (ROS) production could be responsible for the impaired biofilm production in TiO_2

NP–treated pathogens. The synthesized NPs induced considerable reduction in the viability of HepG2 *in vitro* and could prove effective in controlling liver cancer. In summary, the green synthesized TiO$_2$ NPs demonstrate multifarious biological properties and could be used as an anti-infective agent to treat biofilm-based infections and cancer.

INTRODUCTION

In the last decade, the world has witnessed tremendous advancements in the field of nanoscience and its applicability in diverse domains, including academics, industry, and medicine. The distinct physicochemical characteristics and high surface area-to-volume ratio of nanoparticles (NPs) make them attractive candidates for the development of biocompatible materials that can be used in industries and clinical settings (Eisa et al., 2019). Metallic NPs with desired properties have been synthesized using several physical and chemical methods; however, these methods are expensive, utilize hazardous chemicals, require high levels of energy, and expel toxic byproducts that are deleterious to the environment (Patra and Baek, 2015). Therefore, we need methods that exert minimum risk on the environment and also are economically cost effective.

In recent years, bioinspired fabrication of NPs using various biological systems, such as microorganisms (Oves et al., 2019) and plants (Al-Shabib et al., 2016, 2018b), has gained momentum. The plant-mediated NP synthesis has generated lot of interest due to the wide availability of the plants; safe, clean, and eco-friendly synthesis; and low energy consumption (Rajkumari et al., 2019). Aqueous extracts of various plant parts, including seeds, roots, leaves, stems, and fruits have been used for metallic NP synthesis. The phytoconstituents in the extract act as reducing and stabilizing agents for non-toxic NP production (Siddiqi et al., 2018).

The phytomediated synthesis of titanium oxide (TiO$_2$) NPs has great potential in producing anti-infective agents. Titanium oxide NPs are documented to be safe, stable, non-toxic, and having surface activity; hence, they are among the most widely used nanomaterials. The biomediated production of TiO$_2$ NPs has found application in disease treatment, surgical product manufacture, photocatalysis, tissue engineering, agriculture, and cosmetics (Nadeem et al., 2018). Various plants and their parts have been reported for the production of TiO$_2$ NPs, including *Acanthophyllum laxiusculum* (roots), *Aloe barbadensis* (leaves), *Annona squamosa* (peel), *Calotropis gigantea* (flower), *Cicer arietinum* (seeds), and *Dandelion* (pollen) (Marimuthu et al., 2013; Bao et al., 2012; Roopan et al., 2012; Kashale et al., 2016; Madadi and Lotfabad, 2016; Rajkumari et al., 2019).

Withania somnifera, a member of the solanaceae family, is a well-known medicinal plant in Ayurvedic and Unani system of medicine, commonly called as Ashwagandha. The plant has been documented to exhibit medicinal benefits against several ailments, including neurodegenerative diseases, cancer, and chronic diseases The antibacterial activity of

W. somnifera has been explored for many decades. Studies have shown that its extracts demonstrated bactericidal potential against methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, and *Escherichia coli* (Rizwana, 2012). To date, numerous researchers have synthesized NPs using *W. somnifera*. For instance, silver NPs synthesized from the aqueous extract of *W. somnifera* exhibited broad-spectrum antibacterial and antibiofilm activity. A study reported its multiple modes of action, including microbial growth inhibition, cell membrane damage, and reactive oxygen species (ROS) production (Qais et al., 2018). Studies on TiO$_2$ NP synthesis from *W. somnifera* are still scarce, and this probably is the first report on TiO$_2$ NP synthesis from this plant.

In most natural environments, bacteria and fungi prefer to grow in biofilm mode. Microbial biofilms are a complex ecosystem comprising of one or more species embedded in an exopolysaccharide (EPS) matrix (Galié et al., 2018). Formation of biofilm starts with the adherence of the cells to an inert surface and culminates by the formation of cell clusters embedded in EPS matrix secreted by the microbe (Johnson, 2008). Biofilm control and eradication are a major area of concern for environmentalists, food technologists, and clinicians, as it manifests the microbial community resistant to antimicrobials and disinfectants (Baptista et al., 2018). Further, drug-resistant biofilms on medical implants, such as catheters, sutures, and dental implants, lead to severe persistent infection. Further, it makes the treatment more expensive and harassing for the patient (Costerton et al., 2005). Biofilm structures are formed on different artificial surfaces in the food industry, such as stainless steel, glass, and rubber. This leads to pathogenicity, corrosion of metal surfaces, and organoleptic property alteration, which is critical to various agro-based industries (Galié et al., 2018). In addition to conferring resistance to microbes, reports indicated that biofilm formation has a potential etiologic role in cancer development (Rizzato et al., 2019). Experimental evidence has suggested that cancer initiation and development may be a consequence of the pro-oncogenic properties of biofilms formed by invasive pathogenic bacteria (Johnson et al., 2015).

Considering the deleterious effects of biofilms in infections and cancer, we synthesized TiO$_2$ NPs from the root extract of *W. somnifera* and characterized them using various spectroscopic and microscopic techniques. Further, we studied its broad-spectrum antibiofilm potential against *E. coli*, *Pseudomonas aeruginosa*, methicillin-resistant *S. aureus*, *Listeria monocytogenes*, *Serratia marcescens*, and *Candida albicans*. In addition, we also explored the effects of newly synthesized TiO$_2$ NPs on human liver cancer cell line HepG2.

MATERIALS AND METHODS

Collection of Plant Sample and Preparation of Aqueous Extract

The root of *W. somnifera* was obtained from The Himalaya Drug Company, Dehradun, India. The authentication and identification of the plant material were done at Himalaya Drug Company, as well as at the Department of Botany, AMU, Aligarh, and a voucher specimen (WS/R-AGM/HDCO/01-2017) is submitted at the Department of Agricultural Microbiology, AMU, Aligarh, India. A 5% aqueous extract was prepared in of double-distilled water by heating at 100°C for 1 h. The suspension was centrifuged (15,000 × g for 10 min) and filtered to obtain the extract.

Synthesis of Porous TiO$_2$ Nanoparticles (TiO$_2$ NPs)

The fine root powder of *W. somnifera* was washed and dried and then used to make aqueous extract by boiling. The synthesis of TiO$_2$ NPs was carried out using aqueous extract of *W. somnifera* by previously method with slight modifications (Velayutham et al., 2012). The obtained root extract was mixed with titanium (IV) oxide (5 mM) in a round-bottom flask under constant stirring. In the reaction mixture, 1 mM NaOH was added drop-wise and stirred at 70°C for 3 h. The as-prepared white TiO$_2$ NPs NPs were separated by centrifugation (15,000 × g, 20 min), washed thrice with distilled water and then with ethanol, and dried overnight at 120°C to obtain porous fine powder that was further characterized by various structural and morphological techniques. The synthesis of nanomaterial via green route is simple, efficient, facile, inexpensive, and ecofriendly that does not require any special condition, such as vacuum, urbane instrument, catalyst, or template, and so on.

Characterization

X-ray diffraction (XRD) measurements were conducted using a Rigaku Ultima IV diffractometer (Japan) with CuKα radiation (α = 1.54056 Å). Fourier transform infrared (FTIR) spectra were measured with a JASCO spectrometer 4100 (United States) using the KBr pellet technique. The thermal stability of the porous sample was studied using Mettler Toledo thermogravimetric analysis (TGA)/DSC 1 STARe thermogravimetric analyzer (Switzerland) between 50°C and 900°C. The porosity and Brunauer-Emmett-Teller (BET) surface area measurement of the sample were measured at liquid nitrogen temperature with a Micromeritics TriStar 3000 analyzer (Germany) at 77 K. Pretreatment of the samples was done at 200°C for 3 h under high vacuum. Pore-size distributions were calculated using the BJH model on the adsorption branch. Transmission electron micrographs (TEMs) were obtained using a JEOL 2010 microscope (United States) operating at an accelerating voltage of 80 kV. The sample was prepared by placing and evaporating a drop of the sample in ethanol on a carbon-coated gold grid. Scanning electron microscopy (SEM)–energy-dispersive X-ray spectroscopy (EDS) of TiO$_2$ NPs was examined by scanning electron microscopy (SEM,

JSM-7001F; JEOL, United States) equipped with EDS. The NP size distribution and zeta potential results were carried out by dynamic light scattering (DLS) on Malvern zeta potential/particle size analyzer (United Kingdom). The zeta potential values were obtained by applying the Helmholtz–Smoluchowski equation built into the Malvern. Prior to the measurement, 10 mg of the sample was sonicated in distilled water for 10 min. The measurements were repeated three times for each sample.

Microbial Strains and Growth Conditions

Six pathogens, namely, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, *L. monocytogenes* ATCC 19114, *S. marcescens* ATCC 13880, and *C. albicans* ATCC 10231 were used. All bacteria were preserved on nutrient agar at 4°C, and the lone fungus used in the study was maintained in Sabouraud dextrose agar. Bacterial strains were cultured on nutrient broth at 37°C, whereas Sabouraud dextrose broth was used to grow *C. albicans*.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The broth microdilution method using TTC (2,3,5-triphenyl tetrazolium chloride) was adopted to determine the minimum inhibitory concentration (MIC) of TiO$_2$ NPs against all test pathogens. Further, minimum bactericidal concentration (MBC) was determined by macrobroth dilution assay. Test pathogens were grown overnight in media containing TiO$_2$ NPs concentrations (0.5–256 µg/mL) (Qais et al., 2019).

Biofilm Inhibition Assay

Overnight-grown pathogens were diluted in wells containing fresh tryptic soy broth and respective 0.5 × MIC (*E. coli*: 16, *P. aeruginosa*: 32, *L. monocytogenes*: 64, *S. marcescens*: 8, methicillin-resistant *S. aureus*: 32, and *C. albicans*: 64 µg/mL) of TiO$_2$ NPs and incubated at 37°C for a day. After 24 h, the broth was decanted, and the wells were rinsed three times. The cells attached in each well were stained with 1% crystal violet. After 15 min, stain was decanted, and the wells were washed to remove excess stain. Stain was dissolved in ethanol (200 µL), and absorbance was read at 585 nm to determine inhibition (Hasan et al., 2019).

Microscopic Analysis of Biofilm Inhibition

Biofilm inhibition upon treatment with 0.5 × MIC of TiO$_2$ NPs was observed under confocal laser scanning microscope (CLSM) as previously described (Rajkumari et al., 2019).

Quantification of EPSs

Test bacteria cultured in the presence and absence of 0.5 × MIC of TiO$_2$ NPs were centrifuged, and the filtered supernatant was mixed with chilled ethanol and incubated at 4°C for 18 h to

precipitate the EPSs. Phenol–sulfuric acid method to estimate sugars was employed to quantify EPS (Al-Shabib et al., 2018a).

Protein Leakage Assay

In 10 mL growth media, test bacteria, and NPs were added in such a way that the final concentration attained was $0.5 \times$ MIC. The cells were incubated at 37°C with 150 revolutions/min shaking and examined at 0 and 4 h to determine protein leakage. Incubated cells were centrifuged at $18,000 \times g$, and the resulting supernatants were stored at −20°C. Bradford reagent was used to determine the protein concentration in the supernatants (Qayyum et al., 2017).

Disruption of Mature Biofilms

Biofilms of the test pathogens were allowed to grow for 24 h in the wells of a microtiter plate. After incubation, media containing unattached cells was discarded, and adhering cells were incubated again for 24 h in media amended with $0.5 \times$ MIC of TiO₂ NPs. Non-adhering cells were washed with sterile water, and cells bound to walls of the well were stained with crystal violet for 15 min. Absorbance was read at 585 nm after removing the excess stain (Al-Shabib et al., 2018a).

Effect on ROS Production

Intracellularly produced ROS in the test pathogens that were untreated or treated with TiO₂ NPs was determined using an oxidation-sensitive fluorescent probe, 2,7-dichlorofluoroscein diacetate (Qais et al., 2018). The experiment was performed at the respective $0.5 \times$ MICs (*E. coli*: 16, *P. aeruginosa*: 32, *L. monocytogenes*: 64, *S. marcescens*: 8, methicillin-resistant *S. aureus*: 32, and *C. albicans*: 64 µg/mL).

Cytotoxicity Assessment of TiO₂ NPs

HepG2 (ATCC HB-8065) human hepatocellular carcinoma cells and HEK-293 (ATCC CRL-1573) human embryonic kidney cells were obtained from the American Type Culture Collection (Manassas, VA, United States), cultured in Dulbecco modified eagle medium supplemented with 10% fetal bovine serum, 0.2% sodium bicarbonate, and antibiotics at 37°C under humid condition with 5% carbon dioxide, and were used to assess the anticancer potential of TiO₂ NPs. HepG2 cells with 98% viability and passage numbers 20 and 22 were selected for dimethylthiazol diphenyltetrazolium bromide (MTT) and the neutral red uptake (NRU) assays.

MTT Assay

The cell viability of TiO₂ NP–treated HepG2 and non-tumorigenic HEK-293 cells was assessed using yellow dye MTT. Furthermore, cells (10^4) were seeded in 96-well microtiter plate and kept in a CO₂ incubator for 24 h for adherence. Different concentrations of TiO₂ NPs were added, and plate was incubated for another 24 h. MTT (10 µL) was added to each well, and the reaction mixture was kept for 4 h. Dimethyl sulfoxide (200 µL) was added after discarding the supernatant, and absorbance was read at 550 nm (Farshori et al., 2014).

NRU Assay

Neutral red uptake assay was also executed to assess the cytotoxicity employing an earlier reported protocol (Al-Ajmi et al., 2018). Concisely, the medium was aspirated with TiO₂ NPs posttreatment; the HepG2 cells were washed twice and left for 3-h incubation in a medium containing neutral red (50 µg/mL). Solution comprising 0.5% formaldehyde and 1% calcium chloride was used to wash the reaction mixture. The dye was extracted by incubating the cells in a mixture of ethanol (50%) and acetic acid (1%) for 20 min at 37°C. Absorbance was measured at 540 nm.

Statistical Analysis

All experiments were done in triplicate, and data are presented as mean values. The level of significance was analyzed using Student t test in Sigma Plot 12.

RESULTS AND DISCUSSION

Synthesis and Characterization of TiO₂ NPs

The aqueous extract of *W. somnifera* root contains several major active phytochemicals such as withanolides, sitoindosides, amino acids, alkaloids, phenolic compounds, flavonoids, and several other bioactive metabolites (Dar et al., 2015). These compounds act as the stabilizing/capping agent for TiO₂ NPs biosynthesis. Synthesized NPs were characterized using XRD, FTIR, DLS, TGA, BET surface area, SEM, and TEM techniques. In this study, a green approach was deployed for the TiO₂ NPs synthesis using *W. somnifera* extract at room temperature. The TiO₂ suspension was whitish, which changed to light green by the addition of extract, indicating the formation of TiO₂ NPs. The primary characterization of TiO₂ NPs synthesis was done recording the UV-Vis spectra. The bulk TiO₂ showed an absorbance maximum at 440 nm, which blue-shifted to 395 nm after 6 h of reaction, advising the formation of NPs as shown in **Figure 1A** (Kirthi et al., 2011). This agrees with a previous finding where TiO₂ NPs synthesis was confirmed by an absorption peak at 380 nm of UV-Vis spectrum (Murugan et al., 2016).

The phase structure of the synthesized TiO₂ NPs was characterized by XRD. **Figure 1B** presents the XRD pattern of the synthesized sample. The diffraction peaks in the prepared sample can be indexed to the anatase structure phase (JCPDS card no. 21-1272). The synthesized TiO₂ NPs exhibited diffraction peaks at 25.64°, 37.07°, 37.90°, 38.73°, 48.21°, 53.89°, 55.19°, 62.72°, 68.93°, 70.47°, 75.22°, and 76.28°. No feature peaks of rutile (27.45°) were observed, and the Brookite form of TiO₂ NPs and extract/other compounds appeared in the sample. The average crystallite size of the NPs sample was calculated from the anatase FWHM (25.64°) reflection plane using Scherrer formula and found to be 45.28 nm. The anatase diffraction planes were sharp, indicating good TiO₂ NP crystallization.

Fourier transform infrared analysis was performed to assess the role of various phytoconstituents, mainly functional groups, of presence in the extract that were responsible for the capping and stabilization of TiO₂ NPs. The FTIR spectrum of synthesized
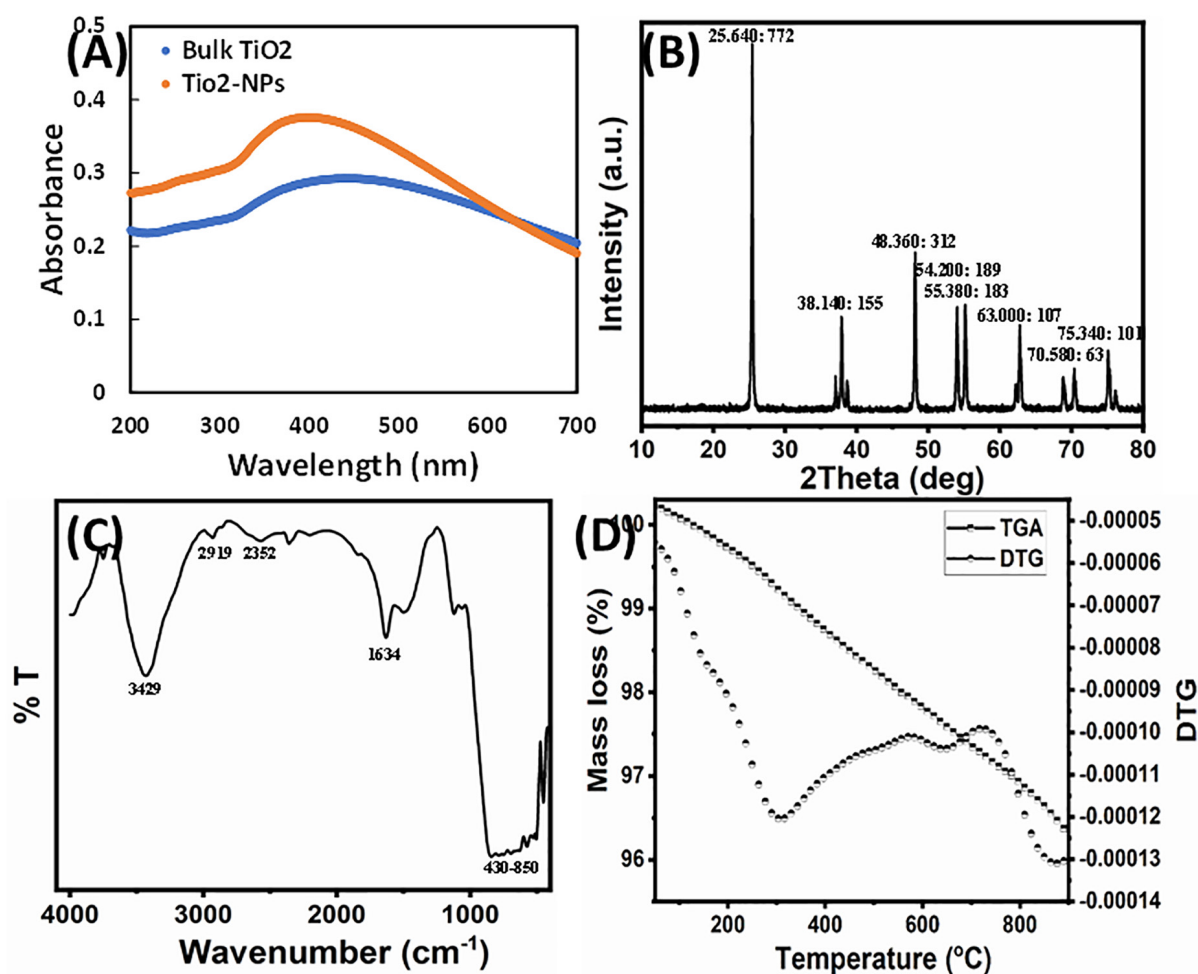
FIGURE 1 | (A) UV-Vis spectra of bulk TiO_2 and TiO_2 NPs. **(B)** X-ray powder diffraction patterns of the TiO_2 NPs. Peak information is provided as (2θ: intensity). **(C)** FTIR spectrum of TiO_2 NPs. **(D)** TG-DTG decomposition curve of TiO_2 NPs.

TiO_2 NPs is shown in **Figure 1C**. A broad and consistent band in IR spectrum of TiO_2 NPs from 430 to 850 cm^{-1} corresponds to the vibration of metal–oxygen (Yan et al., 2004; Amlouk et al., 2006). Moreover, prominent peaks in the 450- to 800-cm^{-1} range are due to Ti–O and Ti–O–O stretching vibrations, confirming the formation of TiO_2 NPs (Sankar et al., 2014; Rajakumar et al., 2015). The formation of TiO_2 NPs was also confirmed by the absorption band near 547 cm^{-1} that corresponds to Ti–O bond (Zhang et al., 2003; Kang et al., 2009). A broadband nearly at 3,429 cm^{-1} is because of the O–H stretching vibration of the interlayer physically absorbing water molecules and of the H–bound OH group (Das et al., 2002). The transmittance band at 2,919 cm^{-1} is due to the vibrational mode of C–H stretching. The peak clearly observed at 1,634 cm^{-1} was assigned to the bending vibration of water molecules (Li et al., 2005). The band observed at 2,352 cm^{-1} was assigned to the existence of CO_2 molecule in air. The presence of reducing sugars and terpenoids in plant extract plays a role in reduction of metal ions and formation of metal NPs (Marchiol et al., 2014). It has been documented that the proteins present in the plant extract

act as a capping agent (Niraimathi et al., 2013). The possible mechanism of capping of the metal NPs in green synthesis is because of the interactions of various phytocompounds present in sufficiently high concentration such as flavanones, terpenoids, alkaloids, and so on, with the particles (Ali et al., 2015). Therefore, the FTIR results indicate that the phytocompounds of root extract of *W. somnifera* were responsible for the synthesis, as well as stabilization and/or stabilization of TiO_2 NPs.

Thermal decomposition performance was carried out in nitrogen gas from 25°C to 900°C and displayed in **Figure 1D**. The TGA curve readily showed three steps of weight loss, as confirmed by the DTG curve. The weight loss up to 900°C of the as-prepared sample is approximately 5.6%. The first weight loss up to 300°C was ascribed to desorption of physically adsorbed/retained water and volatility of the alcohol and acetone solvent. The second weight loss between 300°C and 650°C reflected the elimination of chemically bounded water and the thermal decomposition of plant organic residues. Above 650°C, the weight loss became fairly insignificant, indicating the formation of anatase TiO_2 NPs (Yodyingyong et al., 2011).

The adsorption–desorption isotherm and pore width distribution are presented in **Figure 2A**. It shows that NPs have type IV isotherm with hysteresis loops of H3 (Yu et al., 2010). Type IV adsorption–desorption isotherms indicated the existence of mesoporous entities (4–20 nm, average pore diameter of approximately 24.53 nm), which provide broad surface for biological activity. The plot of differential volumes versus pore diameters indicated a narrower pore-size distribution. It exhibited a specific surface area of about 10.70 m^2/g with specific pore volume of 0.065 cm^3/g.

The DLS graph of TiO₂ NPs are shown in **Figure 2B**. The dynamic light-scattering technique is an efficient method to measure particle diameter and zeta potential in the original grain size distribution. The DLS study indicated the TiO₂ NPs to have an average size of 247 nm, with an intercept of 0.918 and a high–low polydispersity index of 0.631 (**Figure 2Bi**). The zeta potential of the TiO₂ NPs was found to be −24 mV (**Figure 2Bii**) that also evidenced for the stability of TiO₂ NPs. This negative potential was due to a good capping layer of the extract surrounding the NPs (Ravichandran et al., 2016). The size obtained from

DLS analysis was greater than those observed in TEM and SEM because the hydrodynamic diameter was considered in the DLS measurement.

The size and morphology of TiO₂ NPs were characterized by TEM and SEM-EDS. Typical TEM images are shown in **Figures 2Ci,ii**. The TEM results confirmed that the TiO₂ NPs were aggregates of spherical and square NPs, and the size of TiO₂ NPs ranged from 50 to 90 nm. The tendency for agglomeration was caused by van der Waals interactions between individual particles. The TEM image at lower magnification depicted a spherical structure while at high magnification shows the spherical and square shape. The SEM images and EDS spectrum of the sample were taken at 2,000 × magnification, which is shown in **Figures 2Ciii–vi**. It revealed that the overall surface morphology was spherical in shape, porous in nature, and variable in size. The size of NPs varied from 40 to 100 nm, which agreed with the TEM result. The elemental compositions of TiO₂ NPs have been analyzed by EDS, as shown in **Figure 2Cvi**. The elemental compositions revealed that Ti and O were present nearly as
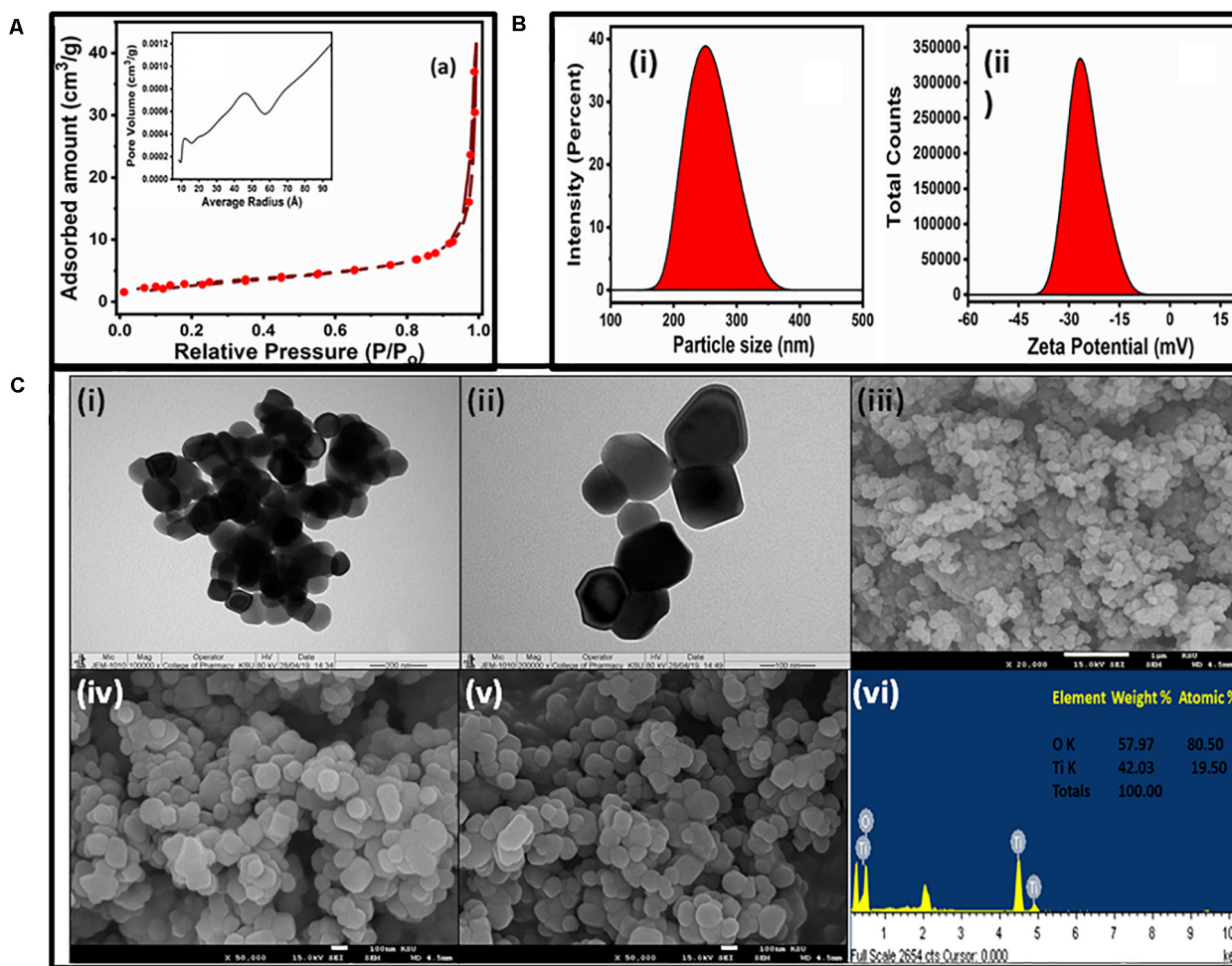
FIGURE 2 | (A) N₂ adsorption–desorption isotherms and pore-size distribution curves (inset) of the TiO₂ NPs. **(B)** Hydrodynamic size **(i)** and zeta potential **(ii)** of the TiO₂ NPs. **(C)** TEM **(i,ii)**, SEM **(iii–v)**, and EDS **(vi)** micrographs of TiO₂ NPs.

per the expected stoichiometry (inset **Figure 2C** vi). The EDS spectrum showed the presence of Ti and O peaks around 4.5 and 0.5 KeV, respectively. The EDS spectrum analysis also revealed that the fabricated TiO₂ NPs were free from any other impurities.

Antibiofilm Activity of TiO₂ NPs

Minimum inhibitory concentration of synthesized TiO₂ NPs was determined against all test pathogens. Among bacteria, the highest MIC value of 64 µg/mL was exhibited by *L. monocytogenes*, whereas *S. marcescens* with MIC of 8 µg/mL was found to be the most sensitive, as shown in **Figure 3A**. Titanium oxide NPs failed to show any bactericidal activity at concentrations lower than 32 µg/mL toward *E. coli*, *P. aeruginosa*, and MRSA. Hence, the 32 µg/mL concentration was considered as the MIC for these three bacteria. Titanium oxide NPs were inhibitory to *C. albicans* at a 64 µg/mL concentration. The MBC values of TiO₂ NPs against test pathogens ranged from 16 to 128 µg/mL as depicted in **Figure 3A**. Similar antimicrobial potential of green synthesized TiO₂ NPs was reported previously (Jayaseelan et al., 2013; Rajkumari et al., 2019).

Drug resistance poses an enormous threat to public health and environment. Biofilms immensely contribute to acquiring and disseminating resistance. These are well-organized multicellular aggregated communities enclosed in a self-secreted envelope of EPSs that prevents antimicrobial diffusion. Further, close proximity and high density of the cells facilitate the transfer of genetic material among the biofilm-making microbes, which is a hotspot for drug resistance (Balcázar et al., 2015). Biofilm-related infections account for spreading various diseases, especially in cases related to medical implants. As far as the food-based industry is concerned, biofilm formation on food matrixes, food contact surfaces, or machines can lead to persistent infections, leading to food-borne diseases (Galié et al., 2018). Hence, microbial biofilm control using eco-friendly NPs is a promising approach in preventing the spread of infection and diseases.

In the current investigation, $0.5 \times$ MIC of TiO₂ NPs was considered to explore its biofilm inhibitory potential against a range of bacteria and *C. albicans*. The results of the biofilm inhibition assay are summarized in **Figure 3B**. Among bacteria, the highest inhibition of 71% was recorded in MRSA, and the lowest was recorded in *L. monocytogenes* (43%). Inhibition of biofilm formation in *E. coli*, *P. aeruginosa*, and *S. marcescens* was observed to be 60%, 51%, and 57%, respectively, compared with the untreated control. Biofilm formation in *C. albicans* was significantly reduced with 32 µg/mL concentration of TiO₂ NP treatment. The percent reduction in biofilm formation was recorded to be 59% over the untreated *Candida* biofilm, as shown in **Figure 3B**. This is probably the first report demonstrating TiO₂ NPs' broad-spectrum biofilm inhibitory activity. Previously, TiO₂ NP have been reported to demonstrate significant biofilm reduction in oral bacteria *Streptococcus mitis* (Khan et al., 2016).

The *in vitro* microtiter plate biofilm inhibition assay results were further confirmed by microscopic analysis. CLSM images showed obliterated biofilm structures in all the pathogens treated with their respective sub-MICs of TiO₂ NPs (**Figure 4**). The untreated control strains showed dense clusters of microbial aggregation, and cells exhibited normal morphology. In contrast, altered biofilm structures were observed in NP-treated cells. Microbial cells were scattered and less dense compared with the untreated control.

Exopolysaccharides are a very important component of the biofilm architecture, as they not only provide structure stability but also protect cells from environmental stresses, entry of antimicrobials, and disinfection (Flemming et al., 2007). Therefore, intrusion in EPS production will certainly have adverse effects on the biofilm-forming capability of the pathogens. We found statistically significant reduction in EPS production of the test pathogens in the presence of $0.5 \times$ MICs of synthesized NPs, as shown in **Figure 5A**. Among the Gram-positive bacteria, MRSA and *L. monocytogenes* showed 81% and 70% decrease in EPS production, respectively, whereas the group of Gram-negative bacteria, namely,

FIGURE 3 | (A) Antibacterial activity of TiO₂ NPs. Bar represents MIC and MBC values in µg/mL of TiO₂ NPs against test pathogens. **(B)** Effect of $0.5 \times$ MIC of TiO₂ NPs on biofilm formation. **significance at $p \leq 0.01$, and ***significance at $p \leq 0.005$.
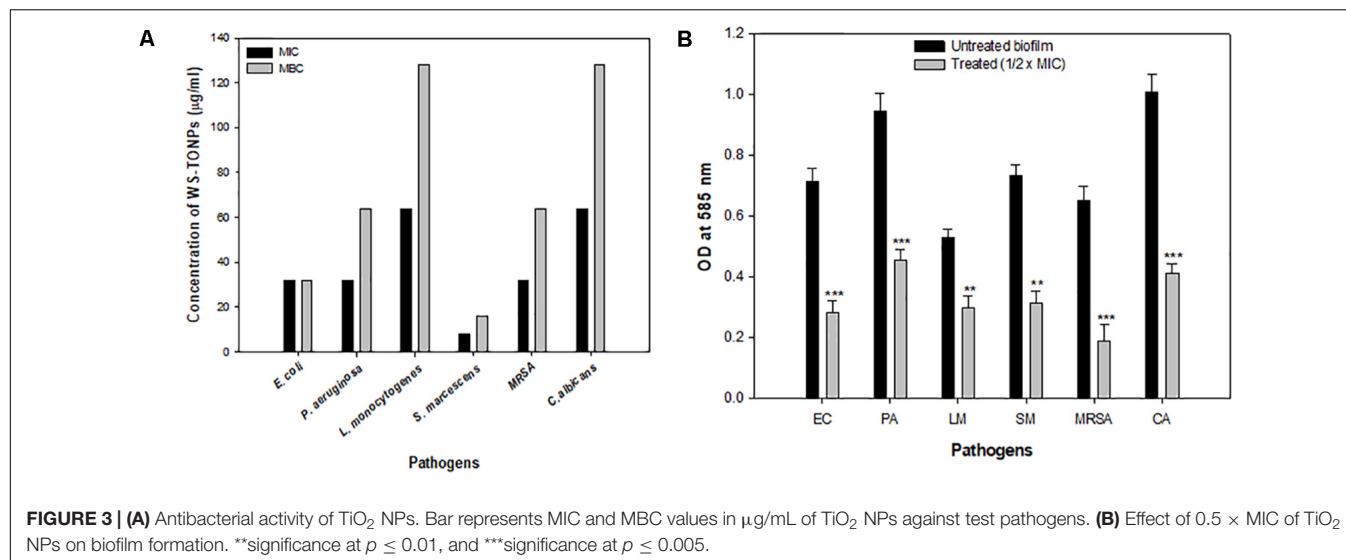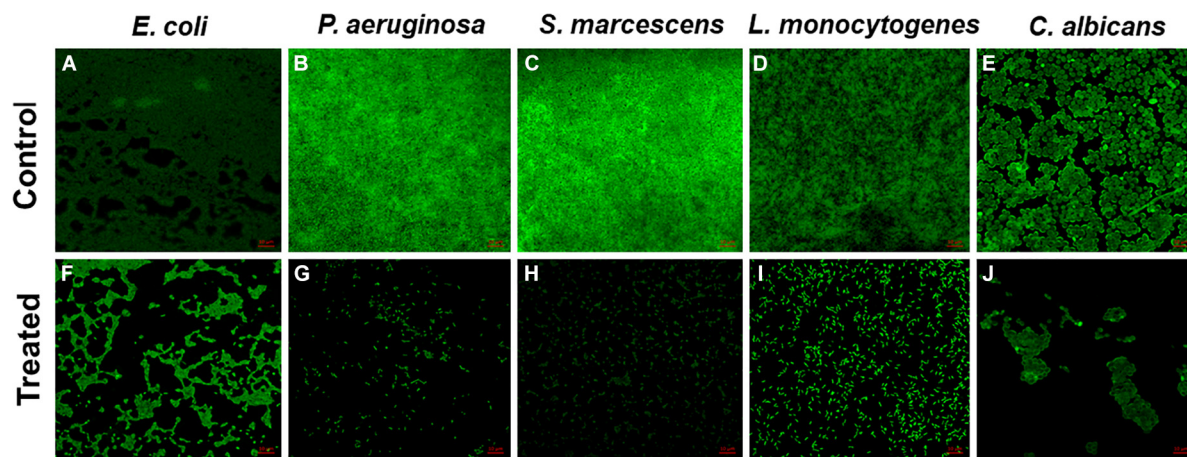
FIGURE 4 | Confocal laser scanning microscopy images. Untreated biofilms of **(A)** E. coli, **(B)** P. aeruginosa, **(C)** S. marcescens, **(D)** L. monocytogenes, and **(E)** C. albicans; inhibition of biofilm by 0.5 × MIC **(F)** E. coli, **(G)** P. aeruginosa, **(H)** S. marcescens, **(I)** L. monocytogenes, and **(J)** C. albicans.
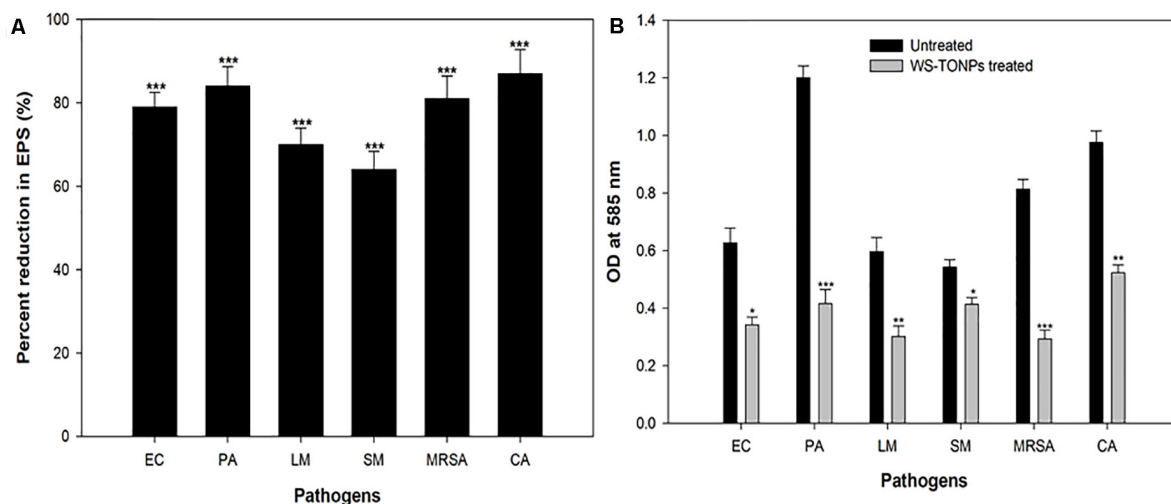
FIGURE 5 | (A) Inhibitory effect of TiO$_2$ NPs on EPS production by test pathogens. ***significance at $p \leq 0.005$. **(B)** Biofilm disruption effect of TiO$_2$ NPs. *Significance at $p \leq 0.05$, **significance at $p \leq 0.01$, and ***significance at $p \leq 0.005$.

E. coli, P. aeruginosa, and S. marcescens, exhibited 79%, 84%, and 64% reduction, respectively. Our findings agree with the researchers who reported reduced EPS production by P. aeruginosa upon treatment with 31.25 µg/mL concentration of TiO$_2$ NPs synthesized from the leaves of A. barbadensis (Rajkumari et al., 2019).

Inhibition of Mature Preformed Biofilms

Mature biofilms are hard to eradicate using chemical agents, owing to the drug resistance that biofilm imparts to the microbial cells residing in this mode. The efficacy of TiO$_2$ NPs in eradicating mature preformed biofilms of the bacterial and fungal pathogens was examined. **Figure 5B** shows histograms depicting the TiO$_2$ NP–induced reduction in the preformed biofilms of the test pathogens. Our data reveal statistically significant disruption of 45%, 65%, 49%, 64%, 24%, and 46% in

E. coli, P. aeruginosa, L. monocytogenes, MRSA, S. marcescens, and C. albicans preformed biofilms, respectively. Biofilm matrix comprising different kinds of biomolecules, such as peptides, polysaccharides, and nucleic acids, is responsible for forming the barrier against antimicrobial agents (Costerton et al., 1999). The assay findings clearly demonstrate that the synthesized NPs could breach the barrier and disrupt the biofilm. For the first time, we reported the broad-spectrum obliteration of bacterial and Candida mature biofilms by TiO$_2$ NPs.

Mechanism of Biofilm Inhibition
Protein Leakage Assay

We performed the leakage assay to study TiO$_2$ NPs' possible mode of action on inhibiting biofilm formation. Significant upsurge in the released protein content in NP-treated samples was observed after 4 h of incubation (**Figure 6A**). These increased

protein content suggested that NPs lysed and destructed the cell wall of the test pathogens, leading to cell death and eventually inhibiting biofilm formation. In a recent report, similar protein leakage due to changes in the membrane permeability of *E. coli* and *S. aureus* cells treated with TiO₂ NPs has been demonstrated (Khater et al., 2020).

ROS Generation Studies

The relative amount of ROS generated in the presence and absence of TiO₂ NPs is summarized in **Figure 6B**. The considerable effect on intercellular ROS production was recorded upon exposure to 0.5 × MIC of TiO₂ NPs. The highest ROS increase of 42% was recorded for *P. aeruginosa*, followed by *C. albicans* (33%), *E. coli* (31%), *S. marcescens* (25%), and MRSA (22%), and the least was recorded for *L. monocytogenes* (19%). ROS generation is one of the chief mechanisms by which NPs interfere with normal microbial cell functions. The ROS-scavenging enzymes present in the bacterial cell neutralize ROS generated in untreated cells, whereas in the NP-treated bacterial cells, enhanced ROS levels overpower the ROS-scavenging enzymes, leading to oxidative stress, which results in lipid peroxidation and, eventually, cell death (Kulshrestha et al., 2017). Hence, we expected that the intracellular ROS produced by TiO₂ NP–treated cells of the test pathogens overpowered the cellular antioxidant defense system and caused cell mortality by inducing oxidative stress. Our results also showed that ROS generation was lower in Gram-positive bacteria, possibly due to their thick cell wall (Al-Shabib et al., 2018a).

Cytotoxicity Studies
MTT and NRU Assay

Anticancer properties of synthesized TiO₂ NPs against HepG2 were assayed by MTT and NRU. Effect on HepG2 cells exposed to different TiO₂ NP concentrations (25–200 μg/mL) is depicted in terms of cell viability (%) in **Figures 7A,B**. Titanium oxide NPs induced concentration-dependent decrease in cell viability of HepG2. Cell viability was recorded as 92%, 88%, 40%, and 26% at 25, 50, 100, and 200 μg/mL concentrations, respectively, using MTT assay (**Figure 7A**). Further, TiO₂ NPs demonstrated insignificant toxicity against non-tumorigenic HEK293 (human embryonic kidney) cells; more than 93% cells were viable at the highest tested concentration of 200 μg/mL (**Supplementary Figure S1**). Similar concentration-dependent cell viability reduction was recorded with NRU assay. The viability of HepG2 cells was found to be 79%, 33%, 25%, and 22% at 25, 50, 100, and 200 μg/mL TiO₂ NP concentrations, in comparison with untreated cells (100%) (**Figure 7B**). The IC₅₀ values obtained were 83.3 and 37.3 μg/mL, respectively, through the MTT and NRU assays.

Microscopic analysis also showed concentration-dependent changes in the morphology of HepG2 cells. Most of the cells at 50–200 μg/mL concentration lost normal morphology, appeared round in shape, decreased cell density, and highly reduced cell adhesion capacity (**Figure 7C**).

Both these assays are sensitive and integrated to measure the anticancer activity of synthesized NPs. The MTT assay evaluates mitochondrial function, whereas NRU assesses lysosomal functions (Ahamed et al., 2017). Possible mechanism of cytotoxicity exhibited by TiO₂ NPs could be the enhanced production of ROS as reported by several workers. Increased ROS levels get the better of the antioxidant defense system, leading to oxidative stress, which triggers apoptosis causing cell shrinkage (Jin et al., 2008; Sha et al., 2011). Similar concentration-dependent HepG2 cell cytotoxicity by TiO₂ NPs synthesized from *Bacillus cereus* has also been demonstrated (Sunkar et al., 2014). Bacterial biofilms formed in human intestines have been reported to sustain and trigger colorectal cancer progression. Molecular processes involved in the interaction of carcinogenic factors formed by pathogens, their biofilms, and the host's response in colorectal cancer initiation and progression have also emerged (Mirzaei et al., 2020). Furthermore, the aggregation of bacteria in biofilms was reported to cause injuries and

FIGURE 6 | (A) Protein leakage from test pathogens treated with 0.5 × MIC of TiO₂ NPs. **(B)** Induction of ROS generation in test pathogens treated with 0.5 × MIC of TiO₂ NPs. *Significance at *p* < 0.05.
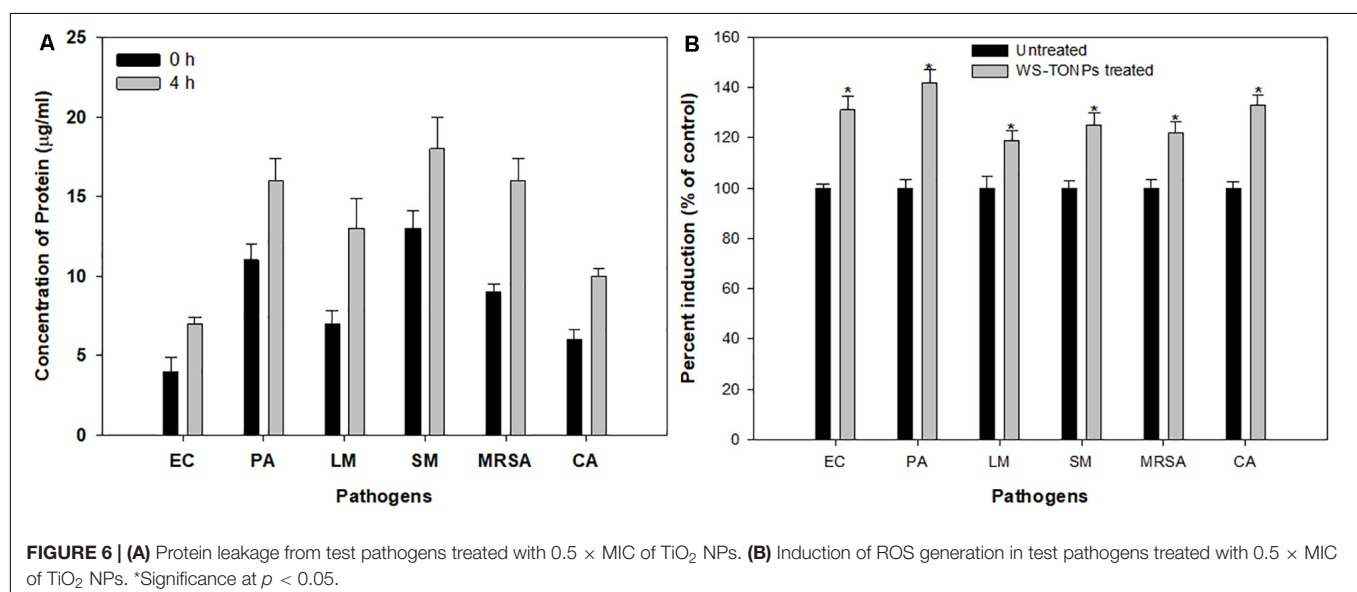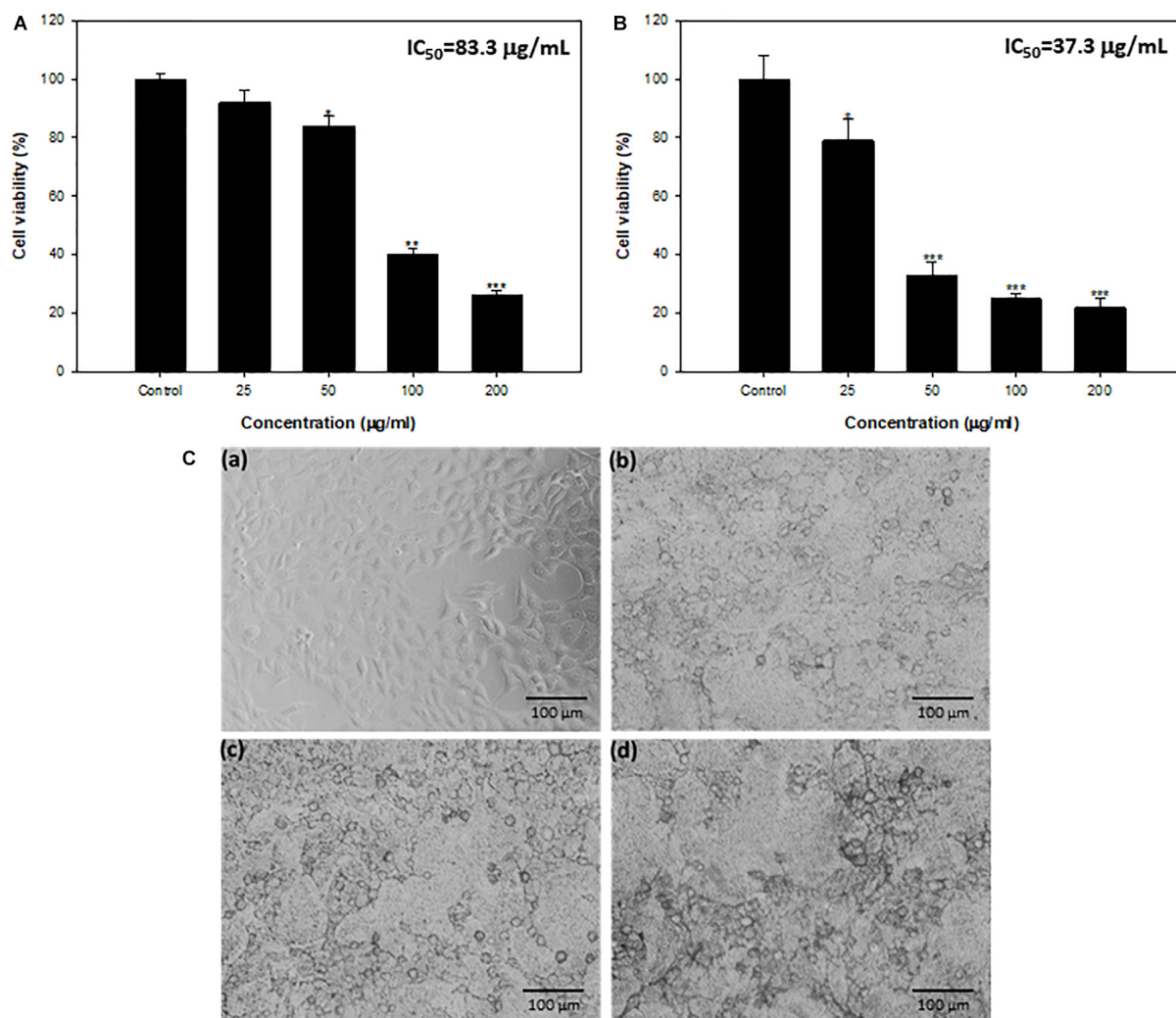
FIGURE 7 | (A) Cytotoxicity determination by MTT assay against HepG2 cell lines. **(B)** Cytotoxicity determination by NRU assay against HepG2 cell lines. *Significance at $p \leq 0.05$, **significance at $p \leq 0.01$, and ***significance at $p \leq 0.005$. **(C)** HepG2 cells exposed to TiO$_2$ NPs for 24 h. **(a)** Control, **(b)** 50 μg/mL, **(c)** 100 μg/mL, and **(d)** 200 μg/mL.

inflammation of intestinal epithelial tissues, thus aggravating the cancer (Gao et al., 2015). Experimental evidence has suggested that initiation and development of cancer are a consequence of pro-oncogenic properties of biofilms formed by invasive pathogenic bacteria (Johnson et al., 2015). Because microbial biofilm is reported to play a critical etiologic role in cancer development, biofilm inhibition, along with TiO$_2$ NP–induced cytotoxicity, is an important finding.

CONCLUSION

In summary, we achieved successful phytomediated synthesis of green TiO$_2$ NPs from root extract of *W. somnifera*, assessed the broad-spectrum biofilm inhibitory activity against bacterial and fungal pathogens, and evaluated HepG2 cytotoxicity. Root extracts of *W. somnifera* acted as a reducing, capping, and

stabilizing agent for the synthesis of TiO$_2$ NPs. Synthesized TiO$_2$ NPs demonstrated significant biofilm inhibition and destruction of preformed biofilms of *P. aeruginosa*, *C. albicans*, *E. coli*, *S. marcescens*, MRSA, and *L. monocytogenes*. Impaired biofilm formation could plausibly be due to the cell death caused by intracellular ROS generation in TiO$_2$ NP–treated pathogens. Furthermore, different concentrations of TiO$_2$ NPs induced significant reduction in HepG2 cancer cell viability. Thus, the synthesized green NPs could prove as effective agents in the treatment of biofilm-based bacterial and fungal infections. Further, these NPs could also have a positive impact in the food industry by reducing environmental biofouling. Moreover, because biofilm formation sustains and triggers cancer development, the cytotoxicity of these NPs against human hepatic cancer cell line HepG2, along with its broad-spectrum biofilm inhibition, can be exploited to prevent and control cancers, with respect to pharmacologic treatments. Finally, more molecular

and animal model investigations are requisite to uncover the exact mechanisms.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

NA-S, FH, FQ, and NA conceived and designed the experiments. FH, FQ, AK, AA, MA, SN, PA, NA, and JK performed the experiments. FH, FQ, NA, MA, SN, PA, TA, JK, AK, and SS performed the experiments and analyzed the data. NA-S, FH, FQ, JK, PA, SN, TA, and AA wrote the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Synergistic Potential of Antimicrobial Combinations Against Methicillin-Resistant *Staphylococcus aureus*

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The chemotherapeutic options for methicillin-resistant *Staphylococcus aureus* (MRSA) infections are limited. Due to the multiple resistant MRSA, therapeutic failure has occurred frequently, even using antibiotics belonging to different categories in clinical scenarios, very recently. This study aimed to investigate the interactions between 11 antibiotics representing different mechanisms of action against MRSA strains and provide therapeutic strategies for clinical infections. Susceptibilities for MRSA strains were determined by broth microdilution or agar dilution according to CLSI guideline. By grouping with each other, a total of 55 combinations were evaluated. The potential synergism was detected through drug interaction assays and further investigated for time-killing curves and an *in vivo* neutropenic mouse infection model. A total of six combinations (vancomycin with rifampicin, vancomycin with oxacillin, levofloxacin with oxacillin, gentamycin with oxacillin, clindamycin with oxacillin, and clindamycin with levofloxacin) showed synergistic activity against the MRSA ATCC 43300 strain. However, antibacterial activity against clinical isolate #161402 was only observed when vancomycin combined with oxacillin or rifampicin in time-killing assays. Next, therapeutic effectiveness of vancomycin/oxacillin and vancomycin/rifampicin was verified by an *in vivo* mouse infection model inoculated with #161402. Further investigations on antimicrobial synergism of vancomycin plus oxacillin and vancomycin plus rifampicin against 113 wild-type MRSA strains were evidenced by combined antibiotic MICs and bacterial growth inhibition and *in vitro* dynamic killing profiles. In summary, vancomycin/rifampicin and vancomycin/oxacillin are the most potential combinations for clinical MRSA infection upon both *in vitro* and *in vivo* tests. Other synergetic combinations of levofloxacin/oxacillin, gentamycin/oxacillin, clindamycin/oxacillin, and clindamycin/fosfomycin are also selected but may need more assessment for further application.

Keywords: synergism, MRSA, vancomycin, combination therapy, *in vivo* model

INTRODUCTION

The inappropriate use and overuse of antibiotics have facilitated the emergence of drug-resistant or even multiple-drug-resistant (MDR) *Staphylococcus aureus* worldwide (Rodríguez-Lázaro et al., 2017). Methicillin-resistant *S. aureus* (MRSA) is a common pathogen for nosocomial infections and exhibits essential resistance to methicillin, oxacillin, nafcillin, carbapenems, and other β-lactams. For now, the clinical therapies against MRSA infection are limited to a few antimicrobial agents, such as ceftaroline, new cephalosporins, retaining significant activity against *S. aureus* and even MRSA strains; and linezolid belonging to the oxazolidinone class and approved for *S. aureus* infections in clinics (Saxena et al., 2019). However, due to the rapid evaluation of antimicrobial resistance, MRSA strains have possessed reduced susceptibilities to vancomycin, daptomycin, levofloxacin, clindamycin, and sulfamethoxazole (Richter et al., 2011). Even worse, the simultaneous resistance to vancomycin, daptomycin, and ceftaroline has been identified in MRSA recently (Wüthrich et al., 2019). Given that the monotherapy is limited in clinical treatment and the new drug development is a lengthy process, the combination therapy has currently become one of the most effective approaches against bacterial infections benefiting from the enlarged spectrum, enhanced antibacterial activity, minimized doses, and reduced drug toxicity of antibiotic combinations. For instance, combination treatments of vancomycin or tigecycline with rifampicin are successful in treatment of many cases (Vergidis et al., 2015). Fosfomycin is a promising option to treat infections caused by multi-drug resistant (MDR) pathogens when combining with daptomycin or β-lactams (Coronado-Álvarez et al., 2019).

In the current study, a total of 11 antibiotics with different mechanisms of antibacterial activity (inhibiting the synthesis of cell wall, protein or DNA, respectively) were selected and combined with each other to examine the pairwise interactions and identify the synergistic combinations against MRSA strains. After the preliminary screening, combinations of oxacillin with levofloxacin, oxacillin with vancomycin, oxacillin with gentamycin, oxacillin with clindamycin, and vancomycin with rifampicin exhibited the collateral effect on the MRSA ATCC 43300 strain. The further experimental verification elucidated that vancomycin combined with oxacillin or rifampicin has synergistic antibacterial activity against the clinical wild-type MRSA strain both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents and Bacterial Strains

Antibiotics of levofloxacin, tigecycline, vancomycin, fosfomycin, linezolid, oxacillin, rifampicin, clindamycin, gentamycin, daptomycin, and chloramphenicol were selected as the representative agents from different categories of antimicrobial agents (**Supplementary Table S1**). The antimicrobial susceptibility testing (AST) of 11 antibiotics was performed according to the CLSI guideline for the 113 clinical MRSA strains isolated from hospitals in Guangzhou, China (CLSI, 2018). The

S. aureus ATCC 29213 was used for the quality control and the MRSA ATCC 43300 was used as the standard strains. The MRSA clinical strain #161402, with multiple resistance to tigecycline, fosfomycin, levofloxacin, oxacillin, rifampicin, clindamycin, and gentamycin, was used in the *in vitro* and *in vivo* experiments to test the therapeutic effectiveness of drug combinations. The Mueller Hinton (MH) broth and agar were used for AST and the Lysogeny broth (LB) and agar were used for drug interaction assays. The Mannitol salt agar (MSA) was used to identify *S. aureus* strains by the gold and yellow color of bacterial colony.

Determination of Single-Drug Concentration

Single-drug concentrations were determined as doses inhibiting bacterial growth. The mid-log cultures of the MRSA ATCC 43300 strain were diluted to 5×10^5 cfu/mL and exposed to LB broth with gradient-diluted antibiotics. The mixtures were incubated overnight at 37°C. After incubation, 200 µL of culture samples were added to 96-well cell incubating plates, and the OD_{600} values were determined using an Ensight^{TM} Multimode Plate Reader (PerkinElmer, Waltham, MA, United States). The OD_{600} value of bacterial growth in drug-free medium was used as the normalization standard. The drug concentrations that were able to inhibit 10–50% of bacterial growth were considered as the potential single-drug concentrations and were used in the following experiments.

Drug Interaction Assays

The interactions of combined antibiotics were investigated as the previous description with minor modification (Yeh et al., 2006). In brief, tubes containing 8 mL of LB broth of mid-log bacterial cultures were mixed with the following four administration options for each combination: (i) 2 mL fresh LB broth as growth control; (ii) 2 mL stock of drug X to measure the growth rate of X singly; (iii) 2 mL stock of drug Y to measure the growth rate of Y singly; and (iv) 1 mL stock of drug X and 1 mL stock of drug Y to measure the combined growth rate. After the overnight incubation, OD_{600} values of all the incubations were determined as described above. The OD_x, OD_y, OD_{xy}, and $OD_{control}$ are representing the groups of drug X and Y singly, the combination of X and Y, and the growth control in the absence of any drugs. The $OD_{control}$ was used as the standard normalization for measuring the growth rates of administration groups, where

$$W_x = \frac{OD_x}{OD_{control}}, \quad W_y = \frac{OD_y}{OD_{control}} \quad W_{xy} = \frac{OD_{xy}}{OD_{control}}.$$

The index of drug interaction ($\tilde{\varepsilon}$) was classified using the following equations as previously described (Yeh et al., 2006):

$$\tilde{\varepsilon} = \frac{(W_{xy} - W_x W_y)}{\left| \widetilde{W_{xy}} - W_x W_y \right|},$$

where $\widetilde{W_{xy}} = \min\left[W_x, W_y \right]$ if $W_{xy} > W_x W_y$, or $\widetilde{W_{xy}} = 0$ if $W_{xy} \leq W_x W_y$;

$$\tilde{\varepsilon} = \frac{(W_{xy} - \min\left[W_x, W_y \right])}{(1 - min\left[W_x - W_y \right])} + 1,$$

where $W_{xy} > \min \left[W_x, \ W_y \right]$.

For $\tilde{\varepsilon} < -0.5$, the interaction is considered as synergistic; $\tilde{\varepsilon} > 0.5$ as antagonistic; otherwise the interaction is scored as additive. A mid-log bacterial density of MRSA ATCC 43300 was used in this experiment, and the concentrations of antibiotics were recommended as above.

In vitro Time-Killing Curves

Illustrated by drug interaction assays, the synergistic combinations (vancomycin/oxacillin, vancomycin/rifampicin, levofloxacin/oxacillin, gentamycin/oxacillin, clindamycin/oxacillin, and clindamycin/fosfomycin) were tested for *in vitro* killing activity against ATCC 43300 and the MRSA clinical isolate #161402. The mid-log cultures of *S. aureus* strains were appropriately diluted to achieve an initial cell density of 10^6 cfu/mL and then exposed to the drug-free, single drug X/Y, and combination of X and Y medium, respectively. The colony counts were then detected and calculated at 3, 6, 9, 24, 27, 48, and 72 h. The concentrations of vancomycin, oxacillin, rifampicin, levofloxacin, gentamycin, clindamycin, and fosfomycin were 2, 1 or 10, 0.03, 0.25, 512, 512, and 320 mg/L respectively, according to the MICs distribution for MRSA strains (**Supplementary Figure S2**).

In vivo Synergism

The neutropenic mouse thigh model was employed for testing the *in vivo* synergistic efficacy of the following drug combinations: vancomycin plus rifampicin and vancomycin plus oxacillin, referring to the considerable synergism against both wild-type and standard MRSA strains upon time-killing curves. The 6-week-old SPF female ICR mice weighing 25 ± 2 g were administered with cyclophosphamide (Yuanye Biotechnology, Shanghai, China) to induce neutropenia (neutrophils $\leq 100/\text{mm}^3$) as previously described (Yu et al., 2019). Briefly, an initial dose of 150 mg/kg of cyclophosphamide was injected intraperitoneally daily for 4 days and followed by a single dose of 100 mg/kg on the fifth day. The mid-log bacterial cultures were appropriately diluted by normal saline, and the neutropenic mice were then intramuscularly injected 100 μL of bacterial suspension (10^7 cfu/mL) into each posterior thigh muscle. After a 1 h, a placebo (normal saline, Group I) or antibiotics was administered in the following manner: single-drug groups received only Drug A (vancomycin, Group II) or Drug B (rifampicin or oxacillin, Group III), and combined groups received both A and B (vancomycin in combination with rifampicin or oxacillin, Group IV). Dosing regimens were 2 mg/kg for vancomycin administrated intraperitoneally, 0.03 mg/kg rifampicin intragastrically, and 1 mg/kg oxacillin subcutaneously, and the injection volume was 100 μL for all drugs. After 24 h, groups of mice were sacrificed, and thigh homogenates in sterile normal saline were sampled for bacterial burden quantifications. In each group, three or four mice were used, and a total of 6 or 8 thigh samples from each group were collected. Both MRSA ATCC 43300 and #161402 strains were tested in this experiment. The significant differences between groups were analyzed using one-way ANOVA, followed by Dunnett's multiple comparisons test using GraphPad Prism 7.0 (La Jolla CA, United States).

Ethics Statement

The SPF female ICR mice were purchased from Hunan Silaikejingda Lab Animal (Hunan, China). Breeding was conducted under SPF conditions. The mice were housed at four per cage with 12-h light:dark cycles and fed SPF food and water *ad libitum*. The *in vivo* mouse study was approved by the Animal Care and Use Committee of South China Agricultural University and followed the Guangdong Laboratory Animal Welfare and Ethics guidelines [GB 14925-2010, SYXK (Guangdong) 2014-0316].

Verification of Combined Antibacterial Effect

To further claim the therapeutic effectiveness of combined antibiotics, sub-inhibitory concentrations of vancomycin (0.5 mg/L) and oxacillin (1 mg/L) and rifampicin (0.03 mg/L) were applied in a series of *in vitro* antibacterial tests against the total 113 wild-type MRSA strains. Firstly, the MICs of vancomycin (in the presence of 1 mg/L oxacillin or 0.03 mg/L rifampicin) and oxacillin (in presence of 0.5 mg/L vancomycin) and rifampicin (in presence of 0.5 vancomycin) were evaluated by agar dilution and compared with single-drug MICs. Secondly, bacterial growth rates in groups of drug-free and monotherapy (oxacillin or rifampicin or vancomycin) and combined therapy (vancomycin/oxacillin or vancomycin/rifampicin) were estimated and calculated. Thirdly, dynamic characteristics of antimicrobial activity were estimated by time-killing curves for 24 h. Details of the procedures were described above. Statistical analysis was assessed using biological replicates ($n = 113$).

RESULTS

The MICs and Single Drug Concentrations

In **Table 1**, The examined 113 clinical strains were highly resistant to levofloxacin showing MIC_{50} and MIC_{90} of 4 and 128 mg/L, oxacillin of 4 and 64 mg/L, clindamycin of ≥ 256 and ≥ 256 mg/L, gentamycin of 64 and ≥ 256 mg/L, and chloramphenicol of 64 and 128 mg/L. MIC distribution of rifampicin showed two sub-populations with MIC <0.125 mg/L and $0.25 \leq \text{MIC} \leq 256$ mg/L, respectively. Antibiotics of tigecycline, vancomycin, linezolid, and daptomycin were susceptible against the most MRSA isolates. MDR strains with resistance to levofloxacin, linezolid, oxacillin, rifampicin, clindamycin, gentamycin, and chloramphenicol were detected in this study as well.

Single drug concentrations that caused 10–50% inhibition of bacterial growth of *S. aureus* ATCC 43300 were evaluated and shown in **Supplementary Table S1** and **Supplementary Figure S1**. For rifampicin, clindamycin, and gentamycin, the maximum of 20% inhibition was observed when given 0.5- to 1-fold MICs. Subinhibitory concentrations of most antibiotics only achieved 30–40% growth reduction, like oxacillin, linezolid,

TABLE 1 | MIC distributions for MRSA strains used in this study.

Antimicrobial agents	≤0.004	0.008	0.015	0.03	0.1	0.13	0.3	0.5	1	2	4	8	16	32	64	128	≥256	MIC$_{50}$	MIC$_{90}$	Resistant breakpoints*
Levofloxacin	0	0	0	0	0	0	4	2	0	37	35	10	3	9	1	4	8	4	128	≥4
Tigecycline	0	0	0	0	0	0	4	9	9	63	20	2	4	1	1	0	0	2	4	NA
Vancomycin	0	0	0	0	0	0	0	0	61	45	7	0	0	0	0	0	0	1	2	≥16
Fosfomycin	0	0	0	0	0	0	0	0	0	2	6	19	38	22	5	2	19	16	≥256	NA
Linezolid	0	0	0	0	0	0	0	21	64	17	0	0	0	0	1	0	2	1	2	≥8
Oxacillin	0	0	0	0	0	0	6	7	14	23	20	22	5	2	4	6	4	4	64	≥0.5
Rifampin	1	13	15	29	3	0	1	1	4	5	8	2	2	3	1	12	13	0.03	≥256	≥4
Clindamycin	0	0	0	0	3	1	0	8	0	0	0	0	0	0	0	2	111	≥256	≥256	≥4
Gentamycin	0	0	0	0	0	1	0	0	3	1	1	1	6	29	26	20	17	64	≥256	≥16
Daptomycin	0	0	0	0	29	55	8	13	0	2	0	1	0	0	0	0	7	0.12	0.5	≤1[a]
Chloramphenicol	0	0	0	0	0	0	0	0	0	1	2	1	9	8	68	16	8	64	128	≥32

| | MIC distribution by the number of isolates (mg/L) |

Resistant breakpoints refer to CLSI documents (M100 2019). [a]Susceptible with MIC ≤ 1 mg/L. NA, not applicable; Red vertical line for breakpoints. Bold values represent the number of strains.

levofloxacin, daptomycin, and chloramphenicol. Notably, the bacteriostatic activity of drugs did not progressively increase with dosing concentrations, which might be due to the characteristics of antibiotics. Sub-MICs of antibiotics used in the drug interaction assays were shown in **Supplementary Table S2**.

Evidence of Synergism

Figure 1 shows the panels of drug interactions which were arrayed in a matrix and painted with colors representing synergistic, antagonistic, or additive effect. Among the total 55 pairwise interactions, 6 combinations exhibited synergistic efficacy against ATCC 43300 ($\tilde{\varepsilon} < -0.5$) and 13 pairwise interactions showed an antagonistic effect ($\tilde{\varepsilon} > 0.5$). Oxacillin exhibited a potential synergism in combination with levofloxacin, vancomycin, gentamycin, and clindamycin. Interestingly, antagonistic buffering and synergistic buffering were also observed in combined interactions indicated as pink and light yellow panels. The additive or indifferent effects were shown by most of the antibiotic combinations as illustrated in white background.

In vitro Effects of Antibiotic Challenge

To test the bacterial responses to drug combinations, killing curves of six pairwise synergistic combinations were evaluated against both the ATCC 43300 strain and clinical MRSA isolate #161402. During the 48 h of exposure, single-drug groups barely exerted the killing activity against either ATCC 43300 or #161402. When administrated with combined drugs, bacterial count reduction of 2–3 log cfu/mL was observed for six pairwise regimens against ATCC 43300. However, combinations of levofloxacin plus oxacillin, gentamycin plus oxacillin, clindamycin plus oxacillin, and clindamycin plus fosfomycin showed insufficient killing activity against isolate #162402. In contrast, the combinations of vancomycin plus oxacillin or rifampin showed considerable inhibition against #161402 at the first 24 h, but regrowth was observed in the following 48 h (**Supplementary Figure S2**). In consideration of the MIC distributions and the *in vitro* killing activity, combinations of vancomycin with oxacillin or rifampicin were selected for the further antibacterial evaluation.

In vivo Synergistic Efficacy

We developed a murine infection model and used ATCC 43300 and #161402 strains to further evaluate the *in vivo* antibacterial efficacy of vancomycin in combination with oxacillin or rifampicin. The bacterial growth in the control groups increased to over 10-log cfu/g at 24 h after inoculation (**Figure 2**) but was inhibited to 7–8 log cfu/g when applying a combination therapy of vancomycin plus rifampicin against both ATCC 43300 and #161402. In addition, the combination of vancomycin plus oxacillin showed a significant decrease in bacterial growth compared with the control groups ($P < 0.001$). Monotherapy of vancomycin, rifampicin, and oxacillin barely inhibited the growth of these two strains, although lower bacterial counts were observed in groups of vancomycin injected alone. Significantly enhanced activity given synergistic combinations

FIGURE 1 | Panels of pairwise combinations for 11 antibiotics. Growth rates of no-drug, drug X only, drug Y only, and combinations of X and Y are shown in each panel (Upper right corner: zoom in on each panel). Error bars represent variability in replicate measurements. Synergism ($\tilde{\varepsilon} < -0.5$), antagonistic ($\tilde{\varepsilon} > 0.5$), and additive ($-0.25 < \tilde{\varepsilon} < 0.5$ and $-0.5 < \tilde{\varepsilon} < 0.25$) effects are labeled with yellow, red, and white backgrounds in the corresponding panels. Panels with pink and light-yellow color represent antagonistic buffering ($0.25 < \tilde{\varepsilon} < 0.5$) and mild synergistic interactions ($-0.5 < \tilde{\varepsilon} < -0.25$), respectively.

was elucidated when compared with the other groups of monotherapy ($P < 0.05$).

Evidence of Synergistic Effect

MICs of vancomycin and oxacillin and rifampicin against 113 wild-type MRSA strains were re-estimated by agar dilution in the presence of the corresponding partnering antibiotics. The magnitude of MIC reduction for each antibiotic when used alone vs. in combination with partners was expressed as a fold change (**Figure 3A**). Notably, when combined with 1 mg/L oxacillin or 0.03 mg/L rifampicin, the MIC of vancomycin dropped nearly 50-fold. On the other hand, the fold-reductions in MICs of oxacillin and rifampicin were >160 and >100, respectively, with an addition of 0.5 mg/L vancomycin. Antimicrobial activity of combinations of vancomycin plus oxacillin and vancomycin plus rifampicin was confirmed by inhibition of bacterial growth (**Figures 3B,C**). When vancomycin combined with oxacillin or rifampicin, the bacterial growth rates were <20% or <40%, which are significantly lower than those of the groups that used single drugs ($P < 0.0001$, one-way ANOVA). In addition, *in vitro* killing curves (**Figure 4**) explained the dynamic antibacterial activity of antibiotic combinations. During 24-h incubation, MRSA strains were inhibited by vancomycin combined with

oxacillin or rifampicin, but a slight regrowth was detected using vancomycin plus rifampicin.

DISCUSSION

Numerous experimental and clinical studies have demonstrated that MRSA strains show basal resistance to methicillin, oxacillin, nafcillin, carbapenems, and other β-lactams (Islam et al., 2019). Currently, MRSA strains are mostly susceptible to vancomycin, daptomycin, and linezolid, the preferred antimicrobial agents for clinical therapies. Primarily by inhibiting the cell wall synthesis, vancomycin shows therapeutic activity against MRSA (Howden et al., 2010). Daptomycin is a cyclic lipopeptide and represents a second generation of glycopeptide antibiotics that are effective against MRSA (Sader et al., 2014). Linezolid has become an important antimicrobial against gram-positive bacteria including MRSA and inhibits the translation by binding to the 23S rRNA peptidyl transferase region (Hashemian et al., 2018). In our study, the MIC distribution indicated that most clinical isolates were susceptible to vancomycin, daptomycin, and linezolid, which was consistent with the previous study (Kates et al., 2018). Similar to the investigation in other countries, we also found high-level

FIGURE 2 | Bacterial densities from mouse thigh muscles (log cfu/g) after 24 h of monotherapy or combination therapy for the two pairwise drugs against ATCC 43300 and #161402, respectively. ****P < 0.001 by the one-way ANOVA.
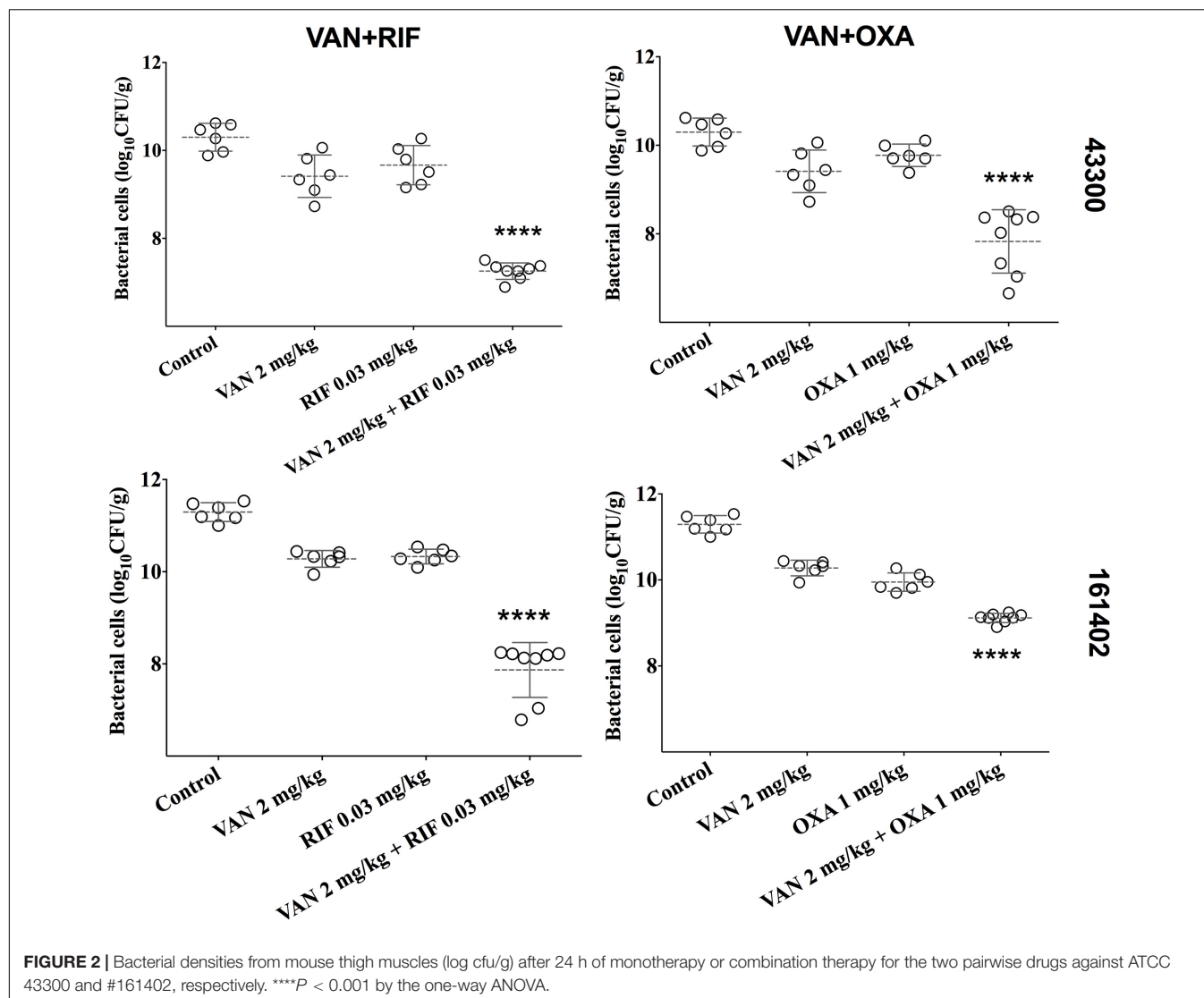
resistance to oxacillin and gentamycin in our tested bacterial population, but MICs of clindamycin and chloramphenicol were different (Sohail and Latif, 2018; Syed et al., 2019). For instance, MRSA isolates from Malaysia, resistant to β-lactams mediated by the PBP$_{2a}$ encoding *mecA* gene, showed high resistance to gentamycin but less to clindamycin (only 31.3%) and moderate resistance to chloramphenicol (Hamzah et al., 2019). On the contrary, the clinical MRSA isolates collected from Guangzhou were highly resistant to clindamycin and chloramphenicol, suggesting a more developed situation of antimicrobial resistance.

By screening the conventional antibiotics used for MRSA infection, we found that vancomycin/oxacillin and vancomycin/rifampicin displayed synergistic effects on MDR-MRSA isolates by both the *in vitro* killing trials and the *in vivo* mouse model. The synergism of vancomycin and the β-lactams has been reported previously as well and achieved significantly lower rates of treatment failure than monotherapy

of vancomycin against MRSA (Truong et al., 2018). Another study demonstrated that the combination of vancomycin and oxacillin showed synergism against three methicillin-resistant vancomycin-intermediate *S. aureus* (VISA) strains and one heterogeneous VISA (hVISA) strain (Pharmaceuticals et al., 2013). The potential synergism may be that vancomycin can easily get into the bacterial cell with the assistance of β-lactams by providing a pathway for entry (Lewis et al., 2018), and the combination of vancomycin and β-lactams down-regulates the expression levels of *mecA* gene in MRSA isolates (Abdolahi and Khodavandi, 2019). In addition, recently, a new theory of collateral susceptibility in antimicrobial agents may inspire a novel insight for the synergism of vancomycin combined with oxacillin. Previous studies reported the oxacillin MICs of *S. aureus* strains decreased after vancomycin treatment (Wang et al., 2017), so called "see-saw phenomenon" occurring in certain stages of vancomycin resistance promotion, suggesting that upon acquisition of vancomycin resistance or VISA evolution, some
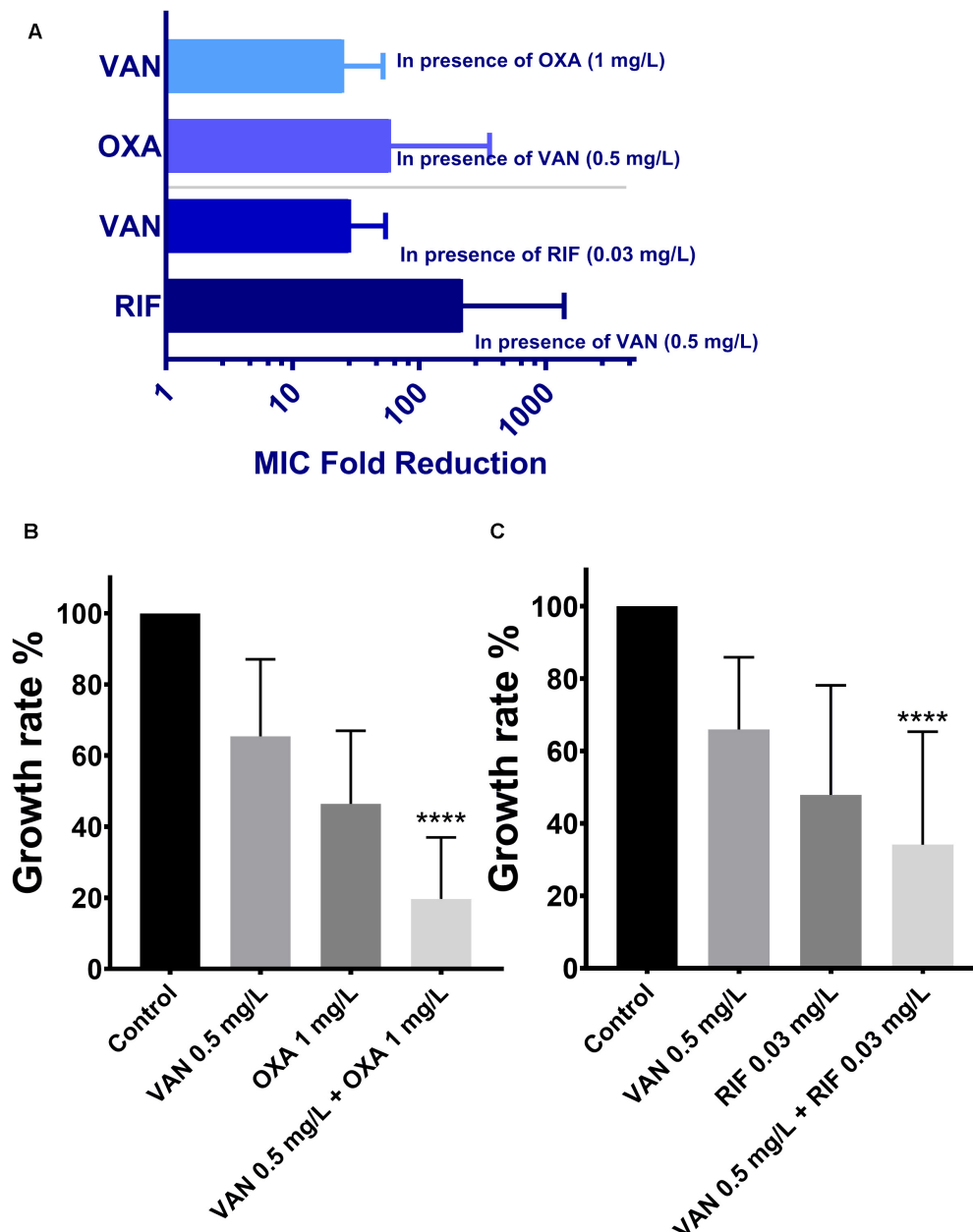
FIGURE 3 | The therapeutic effectiveness of vancomycin combining oxacillin and vancomycin combining rifampicin against the 113 wild-type MRSA strains. The error bars were estimated by 113 biological replicates. **(A)** Fold reduction of MICs in presence of the partnering antibiotics. **(B,C)** Growth rates of drug-free, vancomycin only, oxacillin only, rifampicin only, combination of vancomycin and oxacillin, and combination of vancomycin and rifampicin. ****Significant difference between combination groups and other groups (control and single drug); $P < 0.0001$, one-way ANOVA.

strains show a concomitant decrease in oxacillin resistance (Bhateja et al., 2006). It was reported that mutated *graR* may impair oxacillin resistance (Neoh et al., 2008). The synergistic effect of vancomycin/rifampicin was not only reported in this study, but also in treatment of the non-nosocomial healthcare-associated infective endocarditis (NNHCA-IE) caused by MRSA strain USA 400/SCC mec IV (Damasco et al., 2013). Given that rifampicin interferes with the DNA synthesis while vancomycin disrupts the bacterial cell wall synthesis, the drug combination

may disturb cell reproduction in different stages. For example, when vancomycin combined with rifampicin, significantly higher cell damage and decrease in biofilms thicknesses were detected (Boudjemaa et al., 2017).

As shown in **Figure 1** and **Supplementary Figure S2**, the synergistic activities of levofloxacin/oxacillin, gentamycin/oxacillin, clindamycin/oxacillin, and chloramphenicol/fosfomycin were limited. In the *in vitro* killing curves, these combinations showed no antibacterial activity against clinical
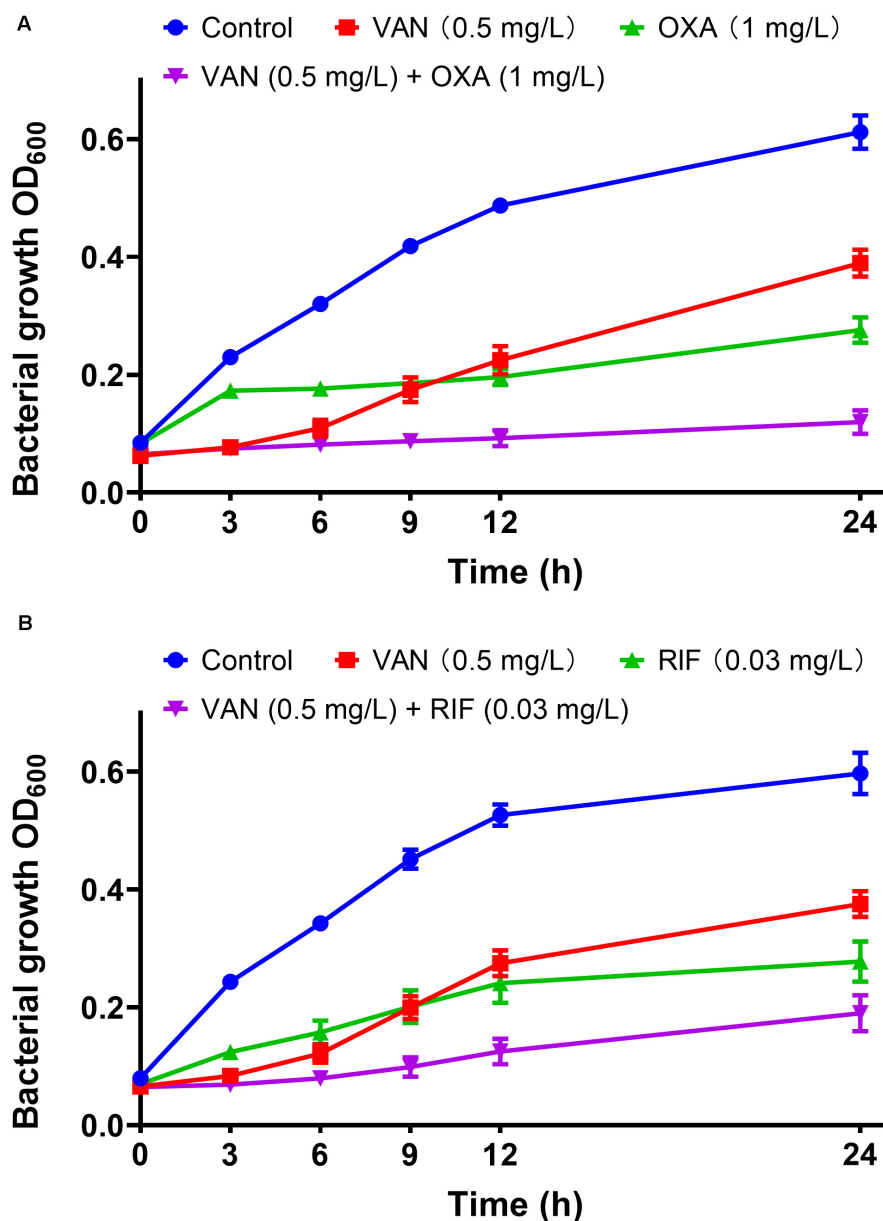
FIGURE 4 | *In vitro* dynamic killing tests of combinations of vancomycin plus oxacillin **(A)** and vancomycin and rifampicin **(B)** against the 113 wild-type MRSA strains. Sub-inhibitory concentrations of 0.5 mg/L (vancomycin), 1 mg/L (oxacillin), and 0.03 mg/L (rifampicin) were used. The error bar was calculated based on the biological repetitions (*n* = 113).

isolate #162402 which was highly resistant to levofloxacin, gentamycin, clindamycin, and fosfomycin with MICs over 256 mg/L. The phenomenon indicated that high resistance to the component of pairwise antibiotics would affect the combined effect, and the ideal situation is that the target pathogens are not highly resistant to either component of the combinations.

In this study, we also found 13 pairwise combinations that showed antagonistic effects ($\tilde{\varepsilon}_{min} > 0.5$) and 10 pairwise interactions that exhibited lower antagonistic effects (**Figure 1**, red and pink panels). However, some of these combinations

showed synergism against MRSA strains in other studies. For example, the combination of daptomycin plus fosfomycin were synergistic in the treatment of experimental endocarditis caused by MRSA strains by both *in vitro* and *in vivo* studies (Garcia-de-la-Maria et al., 2018), and combinations of fosfomycin and rifampin (or tigecycline) have synergistic antibacterial activity in a mouse wound infection model (Simonetti et al., 2018). However, individual differences including serotype, virulence, and antimicrobial resistance must be considered in the evaluation of antibacterial activity, especially for *in vivo* treatment.

CONCLUSION

In conclusion, vancomycin combined with oxacillin or rifampicin was detected as synergistically effective against MRSA infections among a matrix screening of antibiotic combinations. The efficacy of these two combinations was further confirmed by an *in vivo* neutropenic mouse thigh model against a clinical MRSA isolate, suggesting that vancomycin/oxacillin and vancomycin/rifampicin are potential strategies for the treatment of MRSA infections. Studies focusing on the synergism and the mechanism of the combinations should be further investigated for understanding the drug interactions.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the South China Agricultural University.

AUTHOR CONTRIBUTIONS

Y-HL and YY conceived of the study and designed the experiment. X-QY and YY drafted the manuscript. H-LH and J-TF carried out the *in vitro* experiments and analyzed relative data. JS, D-TC, and X-PL revised 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.2020.01919/full#supplementary-material

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Tackling Multidrug Resistance in Streptococci – From Novel Biotherapeutic Strategies to Nanomedicines

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The pyogenic streptococci group includes pathogenic species for humans and other animals and has been associated with enduring morbidity and high mortality. The main reason for the treatment failure of streptococcal infections is the increased resistance to antibiotics. In recent years, infectious diseases caused by pyogenic streptococci resistant to multiple antibiotics have been raising with a significant impact to public health and veterinary industry. The rise of antibiotic-resistant streptococci has been associated to diverse mechanisms, such as efflux pumps and modifications of the antimicrobial target. Among streptococci, antibiotic resistance emerges from previously sensitive populations as result of horizontal gene transfer or chromosomal point mutations due to excessive use of antimicrobials. Streptococci strains are also recognized as biofilm producers. The increased resistance of biofilms to antibiotics among streptococci promote persistent infection, which comprise circa 80% of microbial infections in humans. Therefore, to overcome drug resistance, new strategies, including new antibacterial and antibiofilm agents, have been studied. Interestingly, the use of systems based on nanoparticles have been applied to tackle infection and reduce the emergence of drug resistance. Herein, we present a synopsis of mechanisms associated to drug resistance in (pyogenic) streptococci and discuss some innovative strategies as alternative to conventional antibiotics, such as bacteriocins, bacteriophage, and phage lysins, and metal nanoparticles. We shall provide focused discussion on the advantages and limitations of agents considering application, efficacy and safety in the context of impact to the host and evolution of bacterial resistance.

Keywords: antimicrobial resistance, biofilms, pyogenic streptococci, bacteriocins, bacteriophage, nanoparticles, nanomedicine

INTRODUCTION

The pyogenic group belonging to the genus *Streptococcus* includes species are habitually part of the flora of animals (including humans) and, as such, most species are regarded as commensal, but under fitting circumstances may cause localized and systemic infections (Nobbs et al., 2009; Peters, 2017). Species of the pyogenic streptococci group include *Streptococcus pyogenes*,

Streptococcus agalactiae, *Streptococcus dysgalactiae* subsp. *dysgalactiae* (SDSD), and *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) which, together with *Streptococcus pneumoniae*, are the key pathogens belonging to the genus *Streptococcus* (Parks et al., 2015). For example, *S. pyogenes* is the cause of numerous severe human diseases, including septicemia and streptococcal "toxic-shock" syndrome (Isaacs and Dobson, 2016). *S. agalactiae* is the most frequent cause of sepsis and meningitis in neonates and children (Rajagopal, 2009; Melin, 2011). Considering domestic animals, *S. agalactiae* is one of the main causes of bovine mastitis (Rato et al., 2013). SDSE was primarily considered a human commensal organism but nowadays its relevance as human pathogen is on the raising, causing a similar range of diseases in humans as does *S. pyogenes* (Brandt and Spellerberg, 2009). SDSD has been considered an animal pathogen and is frequently associated with bovine mastitis (Abdelsalam et al., 2013). Human infections associated with this subspecies have been sporadically reported (Koh et al., 2009; Park et al., 2012; Jordal et al., 2015), and its role in human disease remains unclear.

In recent years, severe outbreaks of infectious diseases caused by organisms resistant to multiple antibiotics have occurred. Drug resistance is mounting globally, threatening our capability to treat common infections, resulting in persistent illness and death. It is estimated that by 2050, around 10 million human deaths per year might be attributable to antimicrobial resistance (Neill, 2014, 2016). The increase in antimicrobial resistance is more frightening derived from the considerable narrow number of new antimicrobial agents currently under development (World Health Organization, 2020). The growing of resistance in bacteria has been associated to increased consumption of antimicrobials, and improper prescribing of antimicrobials, leading to selective pressure that trigger drug resistance in exposed bacteria and, consequently, in the persistence of antibiotic resistance genes in populations of the same ecological niches, mainly as a result of horizontal gene transfer (Fair and Tor, 2014). Indeed, high-throughput sequencing and other molecular genetics tools led to a better understanding of the underlying mechanisms of horizontal gene transfer. For instance, in average, about 20% of the fully sequenced genome of *Streptococcus* consists of mobile and exogenous DNA, comprising conjugative and composite transposons, phage regions, and plasmid (Lier et al., 2015; Yamada et al., 2019). Thus, horizontal gene transfer constitutes one of the leading modes of originating gene diversity which confers new antibiotic resistance mechanisms in *Streptococcus*. These gene transfer events frequently strike in the pyogenic group, particularly in *S. pyogenes*, *S. agalactiae*, *Streptococcus canis*, SDSD, SDSE, and *Streptococcus uberis* (Haenni et al., 2010; Richards et al., 2012; Wong and Yuen, 2012; Rohde and Cleary, 2016). Too, there have been reports of an increasing incidence of multiple drug resistance (MDR) among streptococci strains, which hamper customary empirical antimicrobial therapy for these infections. Still, even though pyogenic streptococci remain susceptible to most prescribed antibiotics, treatment failure due to MDR has also been reported both in human and veterinary patients (Doumith et al., 2017; Lai et al., 2017).

The quest for effective approaches to tackle MDR bacteria has put forward several alternatives, such as competitive exclusion of pathogenic bacteria via bacteriocin, and bacteriophages (Rotello et al., 2016; Furfaro et al., 2018; Lopetuso et al., 2019). The effectiveness of some of these new approaches for therapeutics is highly variable, but positive effects have been reported in some species. Irrespective of the mechanism of action, the ways bacteria seem to be able to develop resistance to these new approaches has not received enough attention, making it more difficult to find long-term solutions. Herein, we present an overview of mechanisms of resistance to antimicrobials in pyogenic streptococci, factors that contribute to antibiotic resistance and news approach to treating infectious diseases as an alternative to antibiotics, such as bacteriocins, bacteriophage and phage lysins, and nanoparticles. We shall provide focused discussion on the advantages and limitations of agents considering application, effectiveness, resistance development, and interactions with the immune system.

ANTIBIOTICS AND MECHANISMS OF RESISTANCE

An ideal antimicrobial ought to show high selective toxicity for bacteria with minimal adverse impact to the host (Kohanski et al., 2010). Antibacterial may be organized into four main clusters based on the mechanism of action and target in the bacterial cell – see summary in **Table 1**. Still, the mechanisms of resistance to antimicrobials are complex, and different mechanisms may be present in the same strain promoting a multidrug resistance phenotype, but whose main genotypic and phenotypic characteristics may be schematically grouped as shown in **Figure 1**. Some of these fundamental biochemical mechanisms of antimicrobial resistance include: (i) enzymatic inactivation of antibiotics, e.g., β-lactamases (Munita et al., 2016); (ii) modifications of the antimicrobial target preventing efficient binding of the antibiotic, which often results from spontaneous mutations, including genome and RNA variations (e.g., rRNA mutations associated to resistance to several antibiotics) (Malbruny et al., 2002; Gomez et al., 2017); (iii) preventing drug access to targets, for example through the reduced uptake by the cell via a decrease of outer membrane permeability in Gram-negative and/or active efflux pumps that increase clearance from within the cell (Petchiappan and Chatterji, 2017).

To fully realize the propagation of antibiotic resistance, one needs to recognize the molecular mechanisms of resistance to antibiotics and to map the resistome in different ecological niches. Several studies have assessed the resistome in the environment, namely in wastewater, soil, and gut microbiota of animals (humans included) (Pehrsson, 2016; Von Wintersdorff et al., 2016). Metagenomics directly analyze DNA in a biological sample, allowing for analysis of the resistome within distinct microbial ecosystems (Von Wintersdorff et al., 2016). These studies highlight that determinants of antibiotic resistance, including those clinically relevant, are prevalent in these environments (Lehtinen et al., 2019). Sequence-based studies

TABLE 1 | Mechanisms of the main antibacterial drugs.

Mode of action	Target		Drug examples
Antimetabolites	Folic acid synthesis enzyme	Inhibits enzymes involved in production of dihydrofolic acid	Sulfonamides
		Inhibits the enzymes involved in the production of tetrahydrofolic acid	Trimethoprim
Cell wall synthesis inhibitors	Penicillin-binding proteins	Interact with PBPs and inhibit transpeptidase activity	β-lactams: penicillins (ampicillin, amoxicillin, methicillin penicillin G, penicillin V), cephalosporins (first, second, third, fourth, and fifth generations), monobactams (aztreonam), carbapenems (imipenem, meropenem, doripenem)
	Peptidoglycan subunit transport	inhibits transport of subunits across membrane	Bacitracin
	Peptidoglycan subunit	inhibits the transglycosylation and transpeptidation	Glycopeptides (vancomycin and teicoplanin)
Nucleic acid synthesis inhibitors	RNA	Inhibits RNA polymerase activity	Rifamycin
	DNA	Inhibits the activity of DNA gyrase and, consequently, the DNA replication	Fluoroquinolones: ciprofloxacin, levofloxacin, gatifloxacin, gemifloxacin, garenoxacin, sparfloxacin, and pefloxacin
Protein synthesis inhibitors	30S ribosomal subunit	Promotes mismatches between codons and anticodons, producing defective proteins that are inserted causing disrupt the cytoplasmic membrane	Aminoglycosides: streptomycin, gentamicin, neomycin, and kanamycin.
		Inhibits the interaction of tRNAs with ribosome	Tetracyclines
	50S ribosomal subunit	Blocks peptide bond formation among amino acids	Macrolides: erythromycin and azithromycin. Lincosamides: naturally produced lincomycin and semisynthetic clindamycin. Chloramphenicol.
		Inhibits the formation of the initiation complex between 50S and 30S subunits.	Oxazolidinones: including linezolid

provide large datasets, but one limitation is that they focus on genes already known to be involved in the resistance, or (less frequently) to predict new functions based on the homology to known sequences. These genome annotation schemes will provide more and more information to complement the output of functional metagenomics, which shall result in the identification of new determinants of antibiotic resistance (Von Wintersdorff et al., 2016).

In general, bacterial drug resistance can be divided into intrinsic and acquired resistance (Reygaert, 2018). Intrinsic resistance is a naturally occurring phenomenon, which prevents antimicrobial activity and it is common to the majority of strains of a given species. The intrinsic resistance may be constitutive, i.e., independent of previous antibiotic exposure (e.g., reduced permeability of the outer membrane), or induced via the exposure to antibiotic or environmental stress (e.g., multidrug efflux pumps and biofilm formation) (Baldassarri et al., 2006; Cox and Wright, 2013). Acquired resistance is due to chromosomal point mutations or by acquisition of mobile resistance genes, in which resistant strains emerge from previously sensitive bacterial populations, customarily subsequently to exposure to the antimicrobial (Haenni et al., 2010; Enault et al., 2017).

The acquisition of mobile genetic elements (MGEs), such as bacteriophages, plasmids, integrative and conjugative elements, is recognized as a key point in the emergence of multidrug-resistant (MDR) strains (Lehtinen et al., 2019). The main mechanisms of DNA uptake in bacteria are conjugation, transduction, and transformation (**Figure 2**), which must be followed by recombination to allow stable insertion into the chromosome. These MGEs are self-transmissible elements common in bacteria. Further to genes involved in mobility, regulation, or maintenance, MGEs convey antibiotic resistance genes and virulence factors, such as exotoxins (Haenni et al., 2010). Horizontal transfer of genes (HGT) can modulate host-pathogen interactions and extending the host range. Indeed, the use of high-throughput sequencing tools allowed for a better understanding of HGT. For example, in *S. pyogenes* the lateral exchange of virulence genes, mediated by bacteriophage infection, is a very important factor in the diversification of the species. What is more, bacteriophages may convey genes that provide for selective advantage to the host, thus fostering their own dissemination (Colomer-Lluch et al., 2011; Von Wintersdorff et al., 2016).

Many determinants of resistance are frequently present on a single R plasmid (harboring several antibiotics-resistance genes), thus, multiple resistance can be shared among bacteria in single-event of conjugation (Nikaido, 2009). Many of these R plasmids contain resistance genes against the main classes of antibiotics, such as aminoglycosides, macrolides, phenicol, and tetracycline (Nikaido, 2009).
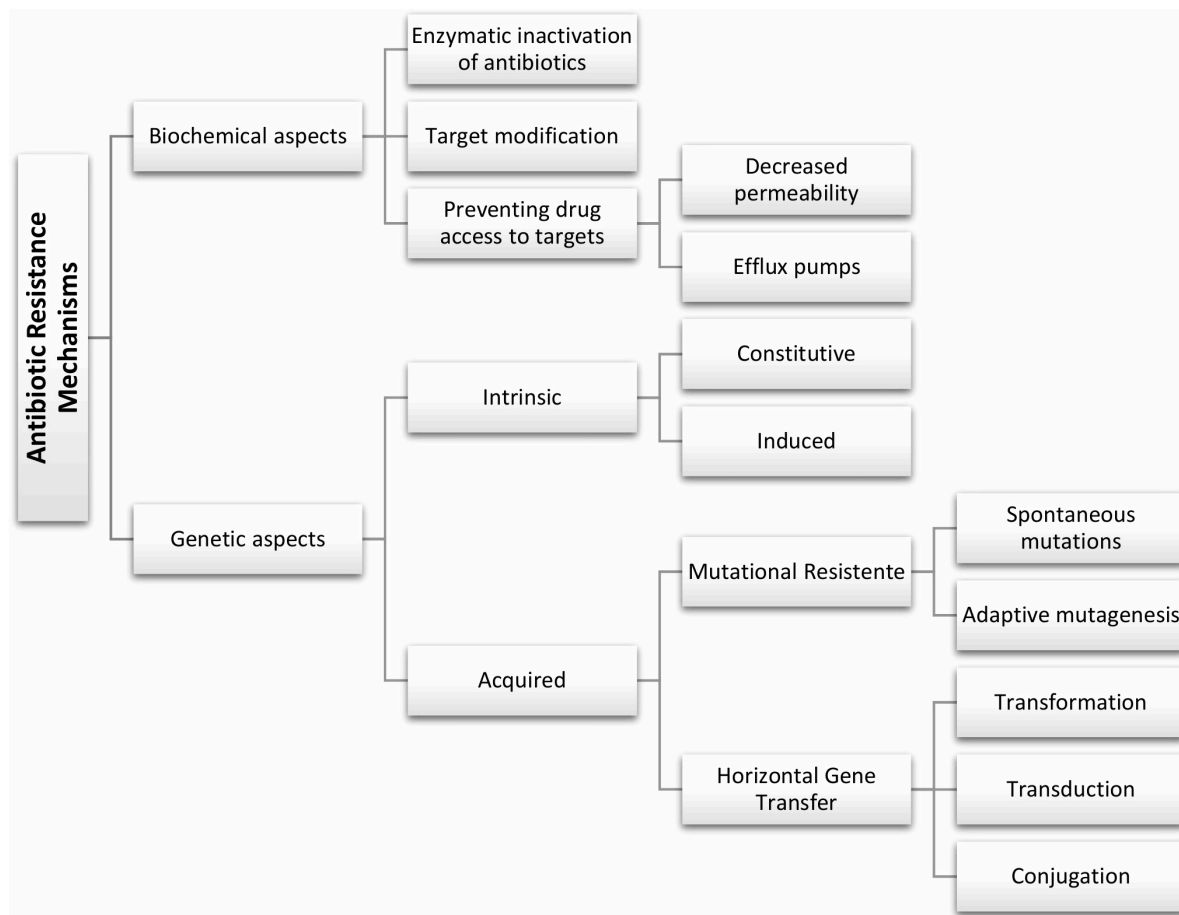
FIGURE 1 | General genotypic and biochemical aspects of antibiotic resistance mechanisms.

Streptococcus harbor various plasmids associated with the transfer of antibiotic resistance and virulence (Grohmann et al., 2003; Cook et al., 2013). In addition to plasmids, a wide variety of transposons have been isolated in streptococci (Brenciani et al., 2007; Fléchard and Gilot, 2014), namely Tn3-family transposons, composite and conjugative transposons. For example, Tn916, encoding *tetM* for the ribosomal protection protein TET(M), associated to independent transfer of resistance between a multitude of strains via a plasmid, including *Enterococcus faecalis*, *Staphylococcus aureus*, *S. pneumoniae*, *S. agalactiae*, and SDSD (Franke and Clewell, 1981; Haenni et al., 2010; Fléchard and Gilot, 2014; Osei Sekyere and Mensah, 2019), that act as reservoirs of functional antibiotic resistance genes.

Several mechanisms of antibiotic resistance among pyogenic streptococci have been reported, whose main mechanism of action and associated resistances shall be briefly described. **Table 2** summarizes the main antibiotics used for the treatment of streptococcal infections and resistance mechanisms.

β-lactams, targeting the bacterial cell wall peptidoglycan, particularly enzymes linked to peptidoglycan synthesis, are one of the most prescribed antibiotics for streptococcal infections due to the broad spectrum of action (Kohanski et al., 2010;

Kong et al., 2010). β-lactamases are secreted enzymes capable of destroying these antibiotics and are the most frequent cause of resistance, but not the only (Bush and Jacoby, 2010; Munita et al., 2016). In fact, pyogenic streptococci have been recognized as non-β-lactamase-producing bacteria, where resistance to β-lactams is essentially mediated by alterations to the binding site of penicillin-binding proteins (PBPs) (Vannice et al., 2019). Nevertheless, a recent study based on whole-genome sequencing revealed the presence of β-lactamases determinants of *S. uberis* and SDSD isolates bovine mastitis (Vélez et al., 2017). Still, there is a need for actional studies to assess the potential of these β-lactamases, and the role of these species as a reservoir of determinants of resistance.

Macrolides are the first choice against streptococcal infections in patients allergic to β-lactam (Kanoh and Rubin, 2010) and, clindamycin (lincosamides) has been used for the treatment of infections associated with anaerobic bacteria as an alternative to penicillin G (Greenwood and Irving, 2012). Three main mechanisms have been associated with resistance to these antibiotics: (i) target modification by methylation of rRNA (*erm* genes) or target mutations, (ii) active efflux, and (iii) enzymatic inactivation
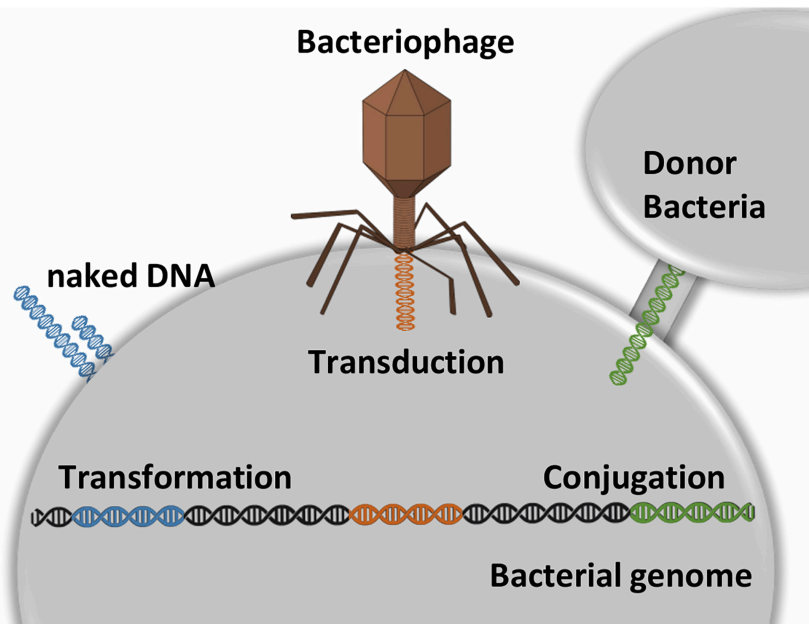
FIGURE 2 | Transformation is the process by which naked DNA from the external environment is incorporated into a bacterial cell. For this process is requires the recipient cell to exhibit on its membrane special DNA binding proteins. Transduction is the process by which a phage transfers DNA from one bacterial strain to another. Conjugation is the process mediated by cell-to-cell contact that provides direct DNA transfer. Conjugative transfer systems associated with plasmids usually code the necessary proteins to DNA exchange. The plasmids are kept as extra-chromosomal genetic material by external selective pressure (e.g., presence of metal or antibiotic). Overall, these mechanisms can be followed by recombination events that allow the genetic determinants to be inserted stably into the chromosome.

(Matsuzaki et al., 2005; Petinaki and Papagiannitsis, 2018). Although most streptococci strains remain sensitive to macrolides and lincosamides, resistance phenotypes have emerged among pyogenic streptococci (Rato et al., 2013; Cattoir, 2016; De Greef et al., 2019).

Since active electron transport is required for aminoglycoside uptake into bacteria, aminoglycosides have weak activity against anaerobic bacteria (Ramirez and Tolmasky, 2010; Krause et al., 2016). Still, low levels of resistance to aminoglycosides are observed in most *Streptococcus* spp., and high-level resistance to aminoglycosides appears to be rare. This resistance occurs due to the production of AAC(6′)-APH(2″), APH(3′)-IIIa, and ANT(6)-Ia enzymes and has been demonstrated to be transferable by conjugation (Cattoir, 2016).

Chloramphenicol-resistant streptococci are not common even though some studies show high levels of resistance among the pyogenic group species, namely, *S. pyogenes*, *S. agalactiae*, and SDSE (Trieu-Cuot et al., 1993; Schwarz et al., 2004; Woodford, 2005). Among streptococci and other Gram-positive bacteria, the resistance to chloramphenicol is mainly mediated by Chloramphenicol *O*-acetyltransferase (CAT) enzymes encoded by plasmids or chromosomally integrated. Several CATs are shared by streptococci, staphylococci, and enterococci strains (Woodford, 2005).

Currently, fluoroquinolones (FQ) have also been put forward as a therapeutic option for the treatment of streptococcal infections (Pinho et al., 2010). However, emergence of resistance among several streptococcal species, including SDSE, *S. pyogenes*, and *S. agalactiae*, *S. pneumoniae*, and viridans group streptococci has been reported (Guerin et al., 2000; Martinez-Garriga et al., 2007; Duesberg et al., 2008; Pinho et al., 2010; Pires et al., 2010; Kimura et al., 2013; Dang et al., 2014; Arias et al., 2019). The most frequent mechanism of high-level FQ resistance is the target modification due to mutations in *parC* and *gyrA* genes that occur mainly in quinolone resistance-determining regions (QRDRs) (Hooper and Jacoby, 2016; Pham et al., 2019). Resistance to FQ can also be mediated by modifying enzymes, target-protection proteins (Pham et al., 2019) and by increased production of multidrug-resistance efflux pumps (Hooper, 2002).

Resistance to tetracyclines (TET) among streptococci strains is often found in high rates (Nakamur et al., 2011; Emaneini et al., 2014; Gherardi et al., 2014; Vélez et al., 2017; Gizachew et al., 2019). In streptococci, genes encoding resistance to TET are frequently acquired by MGEs, which also harbor erythromycin resistance determinants (Brenciani et al., 2004, 2007, 2011; Emaneini et al., 2014; Cattoir, 2016). The presence of determinants of tetracycline resistance (*tet* genes) in conjugative transposons, which can efficiently translocate among related bacteria, may explain the high prevalence of resistance (Santoro et al., 2014). There is a significant association between *tet*M and *erm*B (genetic determinant for erythromycin resistance) that has been identified among the strains of pyogenic streptococci, and it can be co-transferred among *S. agalactiae* and S. *pyogenes* strains (Brenciani et al., 2007; Emaneini et al., 2014). There is also evidence of the linkage between *tet*O and *erm*TR/*mef*A genes (Giovanetti et al., 2003) and lysogenic transfer of these genes

TABLE 2 | Main mechanisms of antibiotic resistance in pyogenic streptococci.

Mechanism	Target antibiotics	Examples	Location	References
Enzyme inactivation	β-lactam	β-lactamases via hydrolisis, e.g., BL2b; TEM-1; TEM-47; TEM-71; TEM-89; TEM-95	Chromosome	Vélez et al., 2017
	Aminoglycosides	Aminoglycoside-modifying enzymes (AMEs) – APH(3′)-IIIa; ANT(6)-Ia; AAC(6′)-APH(2″)	Chromosome and MGE, e.g., Tn4001, Tn5405 and Tn3706 (Tn4001 derivative)	Galimand et al., 1999; Prudhomme et al., 2002; Kristich et al., 2014; Cattoir, 2016; Doumith et al., 2017
	Chloramphenicol	Chloramphenicol O-acetyltransferase (CAT) enzyme encoded by genetic determinants cat(pC194); cat(pC221); cat(pSCS7); catS, and catQ	Plasmid and conjugative transposon Tn1545 and Tn5253-like	Schwarz et al., 2004; Woodford, 2005; Del Grosso et al., 2011; Mingoia et al., 2015
	Lincosamide	LinB/A catalyze adenylylation of antibiotics	MGE	Haenni et al., 2010; Rato et al., 2013; Osei Sekyere and Mensah, 2019
Preventing drug access to target	Tetracycline	Efflux pumps Tet(K)	Chromosomal insertion element	Rato et al., 2013; Emaneini et al., 2014; Nguyen et al., 2014
	Quinolones	Efflux pumps PmrA	Chromosome	Martinez-Garriga et al., 2007
	Macrolides	Efflux pumps Mef and Msr	MGE such as Tn917 and bacteriophage Φ m46.1.	Brenciani et al., 2004, 2007; Di Luca et al., 2010; Del Grosso et al., 2011; Rato et al., 2013; Hadjirin et al., 2014
	Tetracycline	Tet(M), Tet(O), Tet(Q), Tet(S), Tet(T), Tet(W) ribosomal protection proteins dissociate tetracycline from ribosome	MGE (Tn916 and Tn3701) and bacteriophages Φ m46.1	Brenciani et al., 2004, 2007; Liu et al., 2008; Nikaido, 2009; Di Luca et al., 2010; Nguyen et al., 2014; Da Cunha et al., 2014; Silva et al., 2015; Kim et al., 2018
Target modification	Quinolones and Fluoroquinolones	Point mutations primarily in quinolone resistance-determining regions (QRDRs), of parC and gyrA genes	Chromosome. Evidence for horizontal transfer of QRDR between streptococci has been reported.	Ferrándiz et al., 2000; Balsalobre et al., 2003; Orscheln et al., 2005; Pletz et al., 2006; Duesberg et al., 2008; Hooper and Jacoby, 2016; Pham et al., 2019
	Tetracycline; Aminoglycosides	Modification in rRNA	Chromosomal mutation	Nguyen et al., 2014; Lupien et al., 2015
	Macrolides	Modification of 23S rRNA and/or ribosomal proteins L4 and L22 determinants	Chromosomal mutation	Malbruny et al., 2002; Jalava et al., 2004
	β-lactam	Modification of penicillin-binding proteins 1A [PBP1A], PBP2B, and PBP2X	Chromosomal mutation	Kimura et al., 2008, 2013; Pillai et al., 2009; Fuursted et al., 2016; Moroi et al., 2019; Vannice et al., 2019; Musser et al., 2020
	Macrolides-Lincosamide-Streptogramin B	Modification by methylation of rRNA (erm-class genes)	The erm(B) and erm(TR) genes are found in the chromosome of streptococci and conjugative transposons, such as Tn916 family and Tn5397 elements	Brenciani et al., 2007, 2011; Cattoir, 2016

carried by Φ m46.1 among *S. pyogenes* (Di Luca et al., 2010), that contribute to a multi-resistant phenotype.

Due to the rise of pathogens resistant to multiple antibiotics, new strategies have been proposed as an alternative to conventional antimicrobials. One such example is the use of as phage-derived lysins that degrade peptidoglycan (Maciejewska et al., 2018), which may be considered as an alternative to β-lactams, or of bacteriocins that provide a more targeted approach, i.e., strain- or species-specific (Nigam et al., 2014; Matsumoto-Nakano, 2018; Hols et al., 2019). Another emerging field of research has been the use of nanoparticles, particularly metallic nanoparticles (e.g., gold and silver), as direct antimicrobial agents, as drug delivery systems that improve the pharmacokinetics parameters (Masri et al., 2019a), or taking advantage of these nanostructures' optical properties,

e.g., photothermal ablation of cells. The potential of these new approaches against streptococci shall be further discussed in the following sections.

BIOFILMS AND ANTIMICROBIAL RESISTANCE

Generally, bacteria populations may strive as planktonic, i.e., freely existing in solution, and/or sessile forming a biofilm. Biofilms are defined as tri-dimensional agglomerations of cells, attached to biotic or abiotic surfaces, and encased in a self-produced matrix composed by extracellular polymeric substances (Jamal et al., 2018). Their formation might be induced by environmental changes that cause stress cells, such as nutrient

limitation and antimicrobial agents (Garrett et al., 2008; Kumar et al., 2017).

In humans, biofilms account for up to 80% of bacterial infections, according to the United States National Institutes of Health (Khatoon et al., 2018). One of the most important characteristics of biofilms is their ability to increase bacterial tolerance to antimicrobial agents. Biofilms protect the microorganism not only from antimicrobial agents but from nutrients scarcity, mechanical forces, and from the host's immune system. Several *in vitro* studies demonstrated that bacterial biofilm could become 10 to 1,000 times more resistant to the effects of antimicrobials as their planktonic counterparts (Melchior et al., 2006). Therefore, biofilm formation should be considered as a core mechanism of resistance since it increases treatment failure and promotes persistent infection.

Biofilm growth of streptococci has been extensively investigated, but insights in the genetic origin and mechanisms of biofilm formation in this genus are limited. Although most pyogenic streptococci are able to form biofilms, there is substantial heterogeneity among strains in the strength of adherence to different surfaces. Like most bacterial genera, in streptococci biofilms, a gradient of nutrients, waste, and signaling molecules are formed, thus allowing groups of cells to adapt to different environments within the same biofilm, which may be growing at a different rate. Besides that, studies show that a biofilm-specific phenotype is stimulated in a particular subpopulation, resulting in the differential expression of mechanisms against the antimicrobials (Konto-Ghiorghi et al., 2009; Genteluci et al., 2015). Even though the resistance associated to streptococci biofilms are not entirely understood, several mechanisms have been proposed in support of increased resistance to antimicrobials. These mechanisms result from of the multicellular nature of biofilms, which leads to an additive (or synergistic) effect between the biofilm community's protection and the conventional mechanisms of resistance referred above (Rosini and Margarit, 2015; Young et al., 2016).

Formation of biofilms also favors horizontal gene transfer between community members, thus provides conditions for the uptake of resistance genes, e.g., high cell density or accumulation of genetic elements. Some studies suggest that conjugation is more efficient in biofilms than in planktonic cells (Van Meervenne et al., 2014; Kragh et al., 2016). Marks et al. (2014) demonstrated that the biofilm microenvironment of *S. pyogenes* populations results in the induction of competence genes; therefore, it is more conducive to HGT. This study shows for the first time that *S. pyogenes* can be naturally transformed when grown as biofilms.

Overall, upon biofilm formation, there is a delayed internalization of the antimicrobial through the biofilm matrix, as the primary physical and/or chemical diffusion barrier prevents the entrance of polar and charged antibiotics. Additionally, the heterogeneous growth of the biofilm cells and activation of the stress response genes contribute to the resistance phenotype.

The extracellular polymeric substances (EPS) matrix composition is essential for the properties of the biofilm since it offers cohesion and three-dimensional architecture of biofilms (Flemming and Wingender, 2010). The EPS matrix compose 80% of the biofilm containing alginates, poly-*N*-acetyl glucosamine, extracellular teichoic acid, proteins, lipids, nucleic acids, phospholipids, polysaccharides, and extracellular DNA. EPS is 97% of water, which is found as a solvent, dictating viscosity, and mobility (Flemming and Wingender, 2010; Kumar et al., 2017; Jamal et al., 2018). For certain compounds, it is known that the EPS matrix represents an initial barrier, but recent studies showed that the biofilm matrix does not form an impermeable barrier to the diffusion of antimicrobial, and other mechanisms can contribute to promoting biofilm cell survival (Trappetti et al., 2011).

Several reports indicate that the extracellular matrix of pyogenic streptococcal biofilms is rich in proteins (Genteluci et al., 2015; Young et al., 2016; Alves-Barroco et al., 2019). In some cases, the biofilm contains a large amount of mucus-like extracellular component, probably formed by DNA released from dead cells (Alves-Barroco et al., 2019). A role for extracellular DNA was also demonstrated by the reduction of biofilms formed by SDSE isolates after treatment with DNase I (Genteluci et al., 2015). The addition of a carbohydrate oxidant, such as sodium metaperiodate, to the biofilm of SDSE indicated the presence of an exopolysaccharide, like for *Streptococcus mutans* biofilms (Liao et al., 2014) and *Streptococcus intermedius* (Nur et al., 2013). Doern et al. (2009) examined *S. pyogenes* strains from different clinical sources and demonstrated the requirement for protein and DNA in the matrix of biofilm, and only passive role for carbohydrates. This is in contrast to SDSE, for which several polysaccharides have been shown to be required (Genteluci et al., 2015).

Overall, the nature of the biofilm matrix depends on the microbial cells, their physiological status, the nutrients available, and the physical conditions. The composition of the EPS matrix likely influences the resistance against different antimicrobial classes. Responses to specific stress sources such as nutrient limitation the bacterial cell slow its growth. During biofilm development, a gradient is established, in which outer layers are metabolically active and aerobic, while and the more inner layers are anaerobic with the reduced growth rate. This slow growth has been observed in streptococci biofilms that are frequently accompanied to a significant increase in antibiotics resistance (Bjarnsholt et al., 2013; Macià et al., 2014). Several antibiotics, such as aminoglycosides, β-lactams, and fluoroquinolones, do not seem to be active in anaerobic conditions, affecting only the outermost layers of the biofilm (Borriello et al., 2004). Cell-wall active antibiotics, namely, β-lactams and glycopeptide, have minimal activity against bacteria that are not replicating and are metabolically inactive (Del Pozo, 2018).

Clinical strains response to most antibiotics is assessed according to standard MIC determination. However, several studies have indicated that, as a biofilm, the same strain/isolate may be resistant, suggesting that most of the antibiotics evaluated would be ineffective in therapy. Still, information regarding the minimum concentration for biofilm eradication of pyogenic streptococcal is scarce (Conley et al., 2003; Baldassarri et al., 2006).

Biofilm formation of *S. pyogenes* protects against some drug but does not confer complete resistance to some antibiotics, namely, penicillin and fluoroquinolone (Conley et al., 2003; Baldassarri et al., 2006; Young et al., 2016). Therapeutic failures against infections caused by *S. pyogenes* may be due to the ability to internalize human cell and biofilm formation facilitating the persistence of genetically susceptible organisms, additionally supporting the HGT, and consequently, the emergence of virulent clones (Baldassarri et al., 2006). The increased resistance of biofilms to antibiotics was also observed in SDSD and *S. agalactiae* (Mah and O'Toole, 2001; Olson et al., 2002).

As explained above, the successful treatment of infections caused by biofilm-forming bacteria is troubled due to the multidrug-resistant phenotype. Conventional antimicrobial therapy is unable to eradicate the biofilm infection. Consequently, to fight the resistance of bacterial biofilm, several different strategies and antibiofilm agents have been proposed. A promising strategy is the application of nanoparticles, which have been considered as an alternative approach to combat and biofilm-based infections (Baptista et al., 2018). Applications of nanomedicine and other alternative therapies will be discussed below.

ALTERNATIVE ANTIBACTERIAL THERAPIES

In order to tackle the growing MDR concerns, a plethora of alternative compounds, strategies and platforms has been proposed as an alternative to conventional antimicrobials. Some of these alternatives are mere concepts whose promising *in vitro* efficacy has been the focus of attention. Many of these novel solutions have been proposed to be used alone against MDR bacteria, but many other have been proposed to be used in combinatory strategies with traditional antibacterial drugs to enhance efficacy, circumvent the onset of mechanisms of resistance.

Bacteriocins

Bacteriocins are peptides, of prokaryotic origin, with inhibitory activity against diverse groups of microorganisms (Nigam et al., 2014; Hols et al., 2019). Several authors have documented the ability of numerous bacteriocins to inhibit the growth of pathogenic microorganisms. Here we shall refer to a general representation of bacteriocins as an alternative to traditional antibiotics. Overall, bacteriocins interact with the bacterial cell membrane and alter its properties, causing cell death. These molecules normally only target closely related species, and given their bactericidal or bacteriostatic effects, they can offer an advantage relative to conventional antibiotics since treatment could be targeted against specific pathogenic (Lopetuso et al., 2019). These peptides are typically used by commensals microbiota to colonize in the human gastrointestinal tract allowing the survival of specific communities, and thus improving gut barrier function and host immune response (Hols et al., 2019). Four major classes of bacteriocins have been identified: (i) Class I, including small heat-resistant peptides,

modified post-translationally, known as "lanthionine-containing bacteriocins" (e.g., lantibiotics, sactipeptides, and glycocins); (ii) Class II, including small heat-resistant peptides (<10 kDa) post-translational modifications. These are "non-lanthionine-containing bacteriocins" which are divided into four subclasses based on their size; (iii) Class III harboring heat-labile and large proteins (>30 kDa); and (iv) Class IV including complex bacteriocins, namely, large proteins with carbohydrate and/or lipid (Pieterse and Todorov, 2010; Hols et al., 2019).

Widespread applications of bacteriocins have been documented with variable efficacy reports. There has been some experimental evidence supporting the antimicrobial properties of bacteriocin nisin (produced by *Lactococcus*) against relevant oral pathogenic bacteria. It has been shown that nisin A could inhibit the growth of cariogenic streptococci, including *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, and *S. mutans* (Tong et al., 2010). Additionally, it was demonstrated that the nisin associated with poly-lysine and sodium fluoride can inhibit the formation of *S. mutans* biofilms (Tong et al., 2011).

Among bacteriocins used against bovine mastitis, besides the nisin, the lacticin3147 has largely been researched. This bacteriocins has proved effective against the most mastitis-causing pathogens, namely *S. aureus*, SDSD, *S. agalactiae* and *S. uberis* (Ryan et al., 1996, 1998). Studies have shown that bacteriocins produced by several streptococci to be able to inhibit closely related strains (Nigam et al., 2014; Matsumoto-Nakano, 2018; Hols et al., 2019). Some *S. mutans* and *Streptococcus salivarius* strains that are part of the commensal microbiota of the oral cavity are also producers bacteriocin producers (Tagg, 2004; Tagg et al., 2006). Healthy microbiota of the nasopharynx also harbors bacteriocin-producing strains, including *S. salivarius* strains. The bacteriocins produced by this species have been investigated for the treatment of pharyngitis and otitis (Walls et al., 2003). In order to shield against streptococcal infections, bacteriocin-producing strains are inoculated in the nasopharynx (Walls et al., 2003). The ability of normal microbiota strains to inhibit the growth of other bacteria has a critical role in its colonization of the host and suggest that these bacteriocins provide protection against *S. pyogenes* infection (Wescombe et al., 2012).

To date, few streptococci bacteriocins against mastitis-causing pathogens have been identified. However, the natural environment of bacteriocin-producing bacteria consists of a particular field for application. *S. uberis* strains isolated from bovine mastitis bacteriocin-producing has been described, the most studied is the nisin U. This bacteriocin showed activity against important mastitis-causing pathogens, specifically *E. faecalis*, SDSD and *S. agalactiae* (Wirawan et al., 2006).

Larger bacteriocins (above 10 kDa) also produced by some streptococci strains and are identified as bacteriolytic enzymes or non-lytic inhibitory. Examples comprise streptococcin A-M57 produced by *S. pyogenes* and dysgalacticin provided by SDSE. The genes that encode for SA-M57 (*scnM57*) and dysgalacticin (*dysA*) have been found on plasmids pDN571 and pW2580, respectively (Heng et al., 2006). The DysA and ScnM57 are polypeptides with 220 and 179 amino acids, respectively, both are exported via the

Sec-dependent transport pathways. Interestingly, a pW2580-like plasmid is also harbored by some *S. pyogenes* strains, emphasizing the HGT between SDSE and *S. pyogenes* (Heng et al., 2006). Overall, lateral transfer of bacteriocin production underscores the contribution of the microbial ecology within the specific niche.

Nonetheless, the broad use of bacteriocin can also confer threatening for its usage on a large scale. Usually, bacteriocin resistance is acquired by lateral transfer of the immunity gene harbored in bacteriocin-producing strain. Resistance genes located on MGE can facilitate the transfer to closely related or even different species providing the means to resist specific bacteriocins (Dicks et al., 2018).

Multidrug efflux pumps also provide resistance to bacteriocins of several bacterial species (Van Hoang et al., 2011). Furthermore, bacteriocins may be degraded by proteolytic enzymes; consequently, they may not be as stable as conventional antibiotics (Tolinački et al., 2010).

Bacteriophage and Phage Lysins

Bacteriophages (or only phages) are viruses that specifically infect bacteria. The interaction between phage and bacteria usually involves particular receptors located in the cell membranes. Therefore, the phage is a natural killer of bacteria (Ghosh et al., 2019; Lopetuso et al., 2019). For this reason, the bacteriophages and phage proteins, namely enzymes, are extensively studied as a future alternative against bacterial infections.

There are many types of phage viruses, but the vast majority of phages can be distinguished into lytic and temperate. The most common approach for therapy involves lytic phages, which are phages that induce cell lysis, and therefore cause bacterial death (Ghosh et al., 2019), whereas the temperate phages integrate within the host genome (lysogenic conversion) (Di Luca et al., 2010). Typically, in the lytic phage life cycle, after the interaction between tail fibers and the host cell surface receptors, the phage secretes specific enzymes that degrade lipopolysaccharide, peptidoglycan and outer membrane (in Gram-negative) to inject the phage DNA. Subsequently, late genes are expressed and take control of the host cell's to then initiate phage DNA replication. The phage DNA replicated expresses genes that encode proteins necessary for new phage particle assembly, endolysins, and holins for host cell lysis. Finally, the new phage particles are released into the environment.

The most significant factor ensuring the efficacy of phage therapy is its self-replicating nature, which distinguishes them from conventional antibiotics. Therefore, the main advantage of using phages for antibacterial treatment is that it can be administered in a low dose, that is, a small number of phages allows producing more of the particles at the infection site (Maciejewska et al., 2018).

Since their discovery in 1915 by Frederick William Twort, the phages were recognized as potential antibacterial, and due to the facility of administration and absence of side effects, phages were used immediately for antibacterial therapy (oral and topical preparations) (Maciejewska et al., 2018). The discovery and introduction of penicillin in the 1940s led to the practically total abandonment of antibacterial therapy with phage in the western countries (de Almeida and Sundberg, 2020).

However, the benefits of antibiotics were lost considerably with the emergence and dissemination of bacterial resistance. The emergence of infectious diseases caused by multidrug-resistant bacterial generated an essential need for alternatives approaches to traditional antibiotics (Chang et al., 2015; Lehtinen et al., 2019). Along these lines, bacteriophages and phage-derived protein therapy get revitalized.

Since the 1980s, the phage therapy revival in western countries has been considered a possible option for combat antimicrobial resistance (de Almeida and Sundberg, 2020). Many research groups have concentrated on this theme of increasing importance, with Belgium pioneering in studies for the clinical use of phages (Pirnay et al., 2018; Jault et al., 2019). Despite the large potential of phages for antibacterial therapy, a small number of clinical trials have been performed in human patients. Besides that, few clinical trials are accepted by public health authorities, for example, the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) (Rios et al., 2016). In the United States and European Union, the phages and phage-encoded enzymes classified as human therapeutic material are subjected to the same implementation regulations as traditional antibiotics (Maciejewska et al., 2018).

There has been a growing interest in phage-derived enzymes with antibacterial activity, including lysins (degrade peptidoglycan), and depolymerases (that degrade polysaccharide, e.g., capsule, biofilm matrix, and lipopolysaccharide) (Maciejewska et al., 2018). Regarding the application of these enzymes, previous studies, including animal models and clinical trials, showed antibacterial activity and reaffirmed the safety of its use (Lopetuso et al., 2019). Nonetheless, the current legislation limits the use of recombinant enzymes in human therapy, mainly for systemic therapy (Schmelcher et al., 2012).

The potential of phage against biofilm-forming bacteria has been demonstrated. The ability of the bacteria to produce biofilms has been considered the most common reason for failure therapeutic of antibiotics due to the impermeability of the biofilm matrix and the diversity of bacterial cells at different metabolic stages. Studies show that some phages have naturally depolymerases able to degrade the biofilm matrix (Abedon, 2015b). Probably, the depolymerases have evolved in response to polysaccharide of the biofilm matrix that covers the membrane receptor required for the interaction between the phage particle and the host cell and the subsequent attachment. The phages can also infect metabolically inactive bacteria of the biofilm since the receptor is present, but the lytic cycle stays pendent until bacterial metabolism to be active (Pearl et al., 2008). The complete eradication by one phage is rather difficult due to a complex structure of the mature biofilm. A combined action (combined therapy) has been suggested as a valid approach, in which depolymerase that degrades polysaccharides of the matrix allowing the phage or antibiotics to achieve the bacteria (Abedon, 2015a). Phage lysins have been also effectively used to remove bacterial biofilms (Meng et al., 2011; Shen et al., 2013; Rico-Lastres et al., 2015).

Bacteriophages infection occurs through specific protein receptors on the bacterial surface, which is the cause of extreme selectivity of these agents but also their main limitation.

Due to this high specificity, phage therapy is of narrow-spectrum compared to traditional antibiotics whose targets are general pathways and processes common to most bacteria (e.g., protein synthesis). Unlike traditional antibiotics, one particular phage has a restricted number of strains as target, in other words, several and different phages are required to combat one only bacterial species. Moreover, phage-based therapy requires the previous identification of bacteria, causing infection to the isolation of specific lytic phage. Methods to isolate bacteriophages with broad-host-range and modifications to expand the specificity have been the target of several approaches, which could reduce the number of phages needed per species (Fairhead et al., 2017; Hyman, 2019). Moreover, the use of a "phage-cocktail" (composed of strictly lytic phages) can expand the spectrum of action and be administered in combination with other antibacterial agents, thus, increasing the potential of phage therapy.

The success of antibacterial phage-based therapy broadly depends on the patient's immune system (PIS) that may recognize and inactivate viral particles. A low-level of antibodies specific to several viral proteins can naturally exist. Moreover, during phage therapy, the activation mechanisms of the immune response can be triggered; thus, phage may be recognized by the PIS, severely compromising the therapeutic effectiveness (Borysowski et al., 2012).

The activity of antibodies in phage lysines inactivation has also been investigated. Studies have shown that antibodies were effective in reducing the half-life of these enzymes (Rashel et al., 2007; Jun et al., 2014). Nonetheless, modification of lysins by dimerization to broaden their half-life has been investigated (Resch et al., 2011). For example, the ClyS (chimeric endolysin) that showed insensitive to antibodies (Pastagia et al., 2011).

Furthermore, phage particles may undergo denaturation by conformational changes irreversible or reversible. A proposed solution is encapsulation within nanocarriers to become the particles insoluble and protecting them from the digestive and immune system (Balcão et al., 2014; Rios et al., 2018). Another critical question is to get the phage particles to the infection site, given that phages do not have pharmacokinetic properties (Ghosh et al., 2019). One of the biggest concerns of phage-based therapy is the gap in understanding of phage-bacteria-human interaction, namely their safety. Concerning phage therapy in immunocompromised patients, although considered safe, its use may be less effective with more associated risks (Roach et al., 2017).

Another aspect associated with security difficulties is horizontal gene transfer. Although low, there is the possibility of HGT affect the pathogenic potential of co-existing bacterial strains by sharing of antibiotic resistance and virulence genes into the population (Lim et al., 2014). The proper phage selection against a given infection still is a challenging question. Moreover, clinical phage resistance *in vivo* also is a complicated issue.

Recent studies in animal models suggest that bacterial mutations resulting in phage-resistance may enhance the pathogen's fitness in its regular niche within the host (Oechslin, 2018). Experimental data showed that phage-resistance occurs in 80% of researches targeting the intestinal environment and

50% of investigations with a model of sepsis. In human studies, phage-resistance has also been observed.

In the pyogenic streptococcus group, strains can escape to phage attack through several mechanisms, comprising spontaneous mutations of the genes encoding receptor, restriction-modification systems, abortive infection mechanisms, and adaptive immunity mediated by CRISPR-Cas systems (Almeida et al., 2016; Euler et al., 2016). The spontaneous mutation is the principal mechanism emergence of resistance and phage–bacterial coevolution. The mutations may provide resistance by changing the bacterial surface molecules in particular phage receptors, and that also determine phage specificity.

Concerning modification-restriction mechanisms, it is based on its abilities to restrict incoming foreign genetic material and to protect host DNA from restriction through modification (methylation, for example) of specific bases in the DNA sequence. Due to host DNA modification, unmodified sequences are then assumed to be foreign and thus cleaved by restriction endonuclease (Stern and Sorek, 2011). Usually, this mechanism causes the death of phage particles but preserves the host. If the system fails, intruding phages will be replicated and modified by the cell, becoming resistant to restriction. In abortive infection (Abi), the host mechanisms arrest phage development at its different steps, e.g., phage transcription, genome replication, or phage genome assembly. Abi mediated resistance ultimately results in the death of both the bacteriophage and the host. It is a selfless defense mechanism since the host dies, but the surrounding population is benefitted (Stern and Sorek, 2011). Although some of these systems work similarly to toxin-antitoxin systems, Abi systems are vastly diverse, and their modes of action are still not completely understood.

The CRISPR-Cas system consists of a multistep process by which small fragments of foreign nucleic acids (or protospacers) are first recognized and included in the host genome. Afterward, these fragments (or spacers), along with Cas proteins, are used as an adaptive immune system that recognizes, degrades or silences foreign nucleic acids (Bondy-Denomy et al., 2013; Rath et al., 2015). However, phages have acquired mutation-based strategies to evade CRISPR/Cas systems, e.g., losing their spacer sequences or encoding products that target Cas proteins (Stern and Sorek, 2011).

Nanoparticles

Nanomaterials have recently gained great interest due to the variety of applications in biomedicine (McNamara and Tofail, 2017). Nanomaterials comprise a range of constructs, materials and functional systems of particles whose size is between 1 and 100 nm. Particularly, these nanotechnology-based materials have found plenty of applications as alternative tools to traditional antibiotics and, more interestingly, as means to prevent the surge in antibiotic resistance (Baptista et al., 2018). The use of nanoparticles as antibiotic therapy has relied on these nanostructures acting as carrier of drugs, either via integration or incorporation into the nanoformulation, or adsorbed to the surface so as to improve biodistribution and pharmacokinetics, e.g., solubility, controlled drug liberation

and therapeutic effectivity (Gao et al., 2018), the main mechanism of action of metallic nanoparticles in bacteria are described in **Figure 3**. However, several nanomaterials have been proposed as antimicrobials, with particular emphasis metallic nanoparticles (the most recent application of these metallic nanoparticles against *Streptococcus* spp. are shown in **Table 3**).

Some of the value of nanotechnology for antimicrobial therapy relate to modulation of the pharmacokinetic profile, where nanoparticle mediated drug delivery might improve conveyance of the drug to the desired tissue. Furthermore, nanoparticles could be designed to enhance solubility, control the release of

drug and increase clearance from the organism, thus improving the therapeutic window of the cargo drug. Also, some synergistic strategies may be used, such as photothermal ablation of cells, where combination to the "traditional" chemotherapeutic may lead to an increase of efficacy. Nevertheless, there are some limitations to the use of these nanomaterials before their successful translation to clinics. One is the limited amount of data on the use of such systems to tackle infection in *in vivo* models, thus preventing adequate assessment of optimal dose, appropriate administration routes and possible interaction of nanoparticles with cells and tissues, whose toxicological profile is not completely understood (Baptista et al., 2018; Lee et al., 2019).

FIGURE 3 | Different mechanism of action of metallic NPs in bacteria. Nanoparticles induce wide effects in bacterial metabolism by different approaches: (i) ROS generation: Ag, Fe, Cu, and Zn NPs induce ROS (reactive oxygen species), the ROS generated are highly reactive toward biological molecules such as proteins and DNA and interact and damage them. (ii) Damage membrane: Ag and Cu NPs interact with chemical groups of bacterial membrane (sulfate or phosphate) and disturb the normal functions. (iii) Drug and gene delivery systems: Au and Fe NPs could be carrier of gene moieties (DNA/RNA) that interact with bacterial gene, or deliver drug improving some pharmacodynamic parameters. (iv) Ribosome: Au NPs inhibit the union of transfer RNA (tRNA) to ribosome. (v) Photothermal therapy of Au NPs mediated by laser irradiation that disturb the membrane structure. (vi) Bacterial respiration: Ag NPs alter the electronic transport and inhibit the respiratory chain.

TABLE 3 | Application of metallic nanoparticles against *Streptococcus* spp.

Metal	Synthesis method		Bacteria	Highlights	References
Silver	Green synthesis	*Terminalia mantaly* extract	*S. pneumoniae*	The biogenic *Terminalia mantaly*-Ag NPs showed significant antibacterial activity compared to the respective extracts	Majoumouo et al., 2019
		Allium cepa and *Allium sativa* extract	*S. pneumoniae*	AgNPs exhibited antibacterial activity against selected vaginal bacteria	Bouqellah et al., 2019
		Fruit extract of *Prosopis farcta*	*S. pneumoniae*	AgNPs increased the antioxidant and antibacterial activity compared with the extract alone, due to high content in phenolic compounds.	Salari et al., 2019
		Tapinoma simrothi	*S. pyogenes*	AgNPs with effective antimicrobial activity in a wide range of bacteria	Sholkamy et al., 2019
	Chemical synthesis	Silver nitrate reduced by sodium borohydrate	*S. pyogenes*	AgNPs as carrier of new quinazolinone compounds showed enhanced antibacterial activity	Masri et al., 2019a
Gold	Green synthesis	*Justicia glauca* extract	*S. mutans*	AuNPs coated with antibiotic increased efficacy against a broad range of bacteria	Emmanuel et al., 2017
		Resveratrol as a green reducing agent	*S. pneumoniae* *S. pyogenes*	AuNPs-resveratrol increased efficacy against *S. pneumoniae* compared to resveratrol	Park et al., 2016
	Chemical synthesis	Reduction of gold (III) chloride trihydrate by sodium citrate	*S. pneumoniae*	Uptake of AuNPs by *S. pneumoniae* associated the antibacterial activity to the formation of inclusion body of AuNP (IB-AuNPs), composed by proteins, carbohydrates and lipids. Some proteins associated with IB-AuNPs could be used for new strategies	Ortiz-Benítez et al., 2019
		Citrate reduction of gold (III) chloride trihydrate	*S. mutans*	Combination of AuNPs and diode irradiation decreased CFUs	Sadony and Abozaid, 2020
Gold-silver	Gold-silver nanocages via galvanic replacement reaction		*S. mutans*	Au-Ag nanocages promoted the inhibition of *S. mutans*	Wang et al., 2016
Gold-titanium	Commercial TiO$_2$ nanotubes with Au via direct current plasma sputter		*S. mutans*	Ti nanotubes sputtered with Au nanorod irradiation increased the inhibitory effect against *S. mutans*	Moon et al., 2018
	Green synthesis: Terminalia chebula bark extract		*S. pneumonia*	Au-TiNPs loaded with carbon nanotubes and irradiated under visible light showed higher antimicrobial activity than ampicillin	Karthika and Arumugam, 2017
Zinc-silver	Polymeric precursor and coprecipitation		*S. mutans*	Zn-AgNPs inhibit *S. mutans* biofilm formation (dentistry)	Dias et al., 2019
Iron	Green synthesis	*Agrewia optiva* and *Prunus persica* extracts	*S. mutans* *S. pyogenes*	FeNPs provided antimicrobial activity and antioxidant capacity associated to compounds from extracts	Mirza et al., 2018
	Chemical synthesis	Commercial NPs	*S. mutans*	Chitosan coated FeNPs as carrier for chlorhexidine; bacteria eradication and antibiofilm effect	Vieira et al., 2019
		Ferric chloride and ferrous chloride tetrahydrate	*S. mutans*	FeNPs on surface for eradication of *S. mutans*	Javanbakht et al., 2016
		Solvothermy employing iron (III) chloride	*S. mutans*	Vitamin B$_2$ coated FeNPs promoted antibacterial activity	Gu et al., 2020
Copper	Chemical methods: copper acetate as precursor		*S. mutans*	Hybrid Cu-chitosan NPs reduced MIC and minimum bactericidal concentration	Covarrubias et al., 2018
	Commercial NPs		*S. mutans*	CuNPs added to orthodontic composite inhibited the growth of *S. mutans*	Toodehzaeim et al., 2018
Zinc	Zinc acetate dihydrate as precursor		*S. pneumoniae*	ZnNPs reduced IMC and showed anti-biofilm formation activity	Bhattacharyya et al., 2018
	Green synthesis: *Costus igneus* extract as capping and reducing agent		*S. mutans*	ZnNPs showed a dose dependent antibacterial and antibiofilm effect against *S. mutans*	Vinotha et al., 2019

Silver Nanoparticles

Traditionally, Silver (Ag) has been employed as antimicrobial, namely silver sulfadiazine and silver nitrate (Lansdown, 2006), which relied on the release of Ag+ that triggers a range of processes resulting in hampered bacterial growth. Silver nanoparticles has allowed improved release of silver ions and, thus, enhance the bactericide action (Dakal et al., 2016). Silver nanoparticles (AgNPs) may be synthesized by different protocols relying on thermic vaporization, chemical or photochemical reduction of silver ions to form nanoparticles that are then capped either by the same reagent or by additional compounds, which promote solubility and stability (Lee and Jun, 2019). The most used method is based on using citrate as reducing and capping agent of silver salts. Nowadays, other methodologies using biological extracts (e.g., plant extracts) has been employed for the "green synthesis" of this nanomaterial (Masooleh et al., 2019). Regardless of the synthesis method, the antibacterial activity of AgNPs depends on the size and shape of the particles; for example, the antibiotic effect increases with smaller sizes due to the dramatic increase in surface area available for ion release and/or to interact with the bacteria. In fact, some authors propose that AgNPs between 1 and 10 nm could interact more efficiently with the bacteria cell membrane (Morones et al., 2005), and spherical nanoparticles seem to be more effective in bacterial eradication than triangles or cylinders (Raza et al., 2016). Still, for AgNPs, size is the stronger determinant associated with antibiotic activity, which is clear in conceptual studies of efficiency for *Pseudomonas aeruginosa* and *Escherichia coli* eradication (Raza et al., 2016). However, AgNPs have shown biocidal effects against a range of bacterial species with clinical interest, such as *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Vibrio cholerae*, and *Salmonella* spp. (Morones et al., 2005; Sánchez-López et al., 2020).

Although the biocidal action of AgNPs has been attributed mainly to the Ag+ release, the actual mechanism is not yet completely elucidated. Crucial to their effect is the fact that AgNPs tend to accumulate at the membrane where they progressively aggregate, allowing silver ions to interact with different functional groups, such as sulfate or phosphate, and disturb the function of the bacterial membrane, promoting its rupture and liberate the cytoplasmatic content (Le Ouay and Stellacci, 2015). Other studies have suggested that Ag+ is able to interact and inactivate some biological structures and affect the bacteria' respiratory process, namely inhibiting the respiratory chain (Costa et al., 2010). However, perhaps the most widely accepted hypothesis is the production of reactive oxygen species (ROS), like superoxide or hydrogen peroxide, which interact with the lipids, proteins or DNA, promoting alteration in the normal functions, triggering lysis and cell death (Quinteros et al., 2016).

AgNPs have also found a range of industrial applications that require some level of inhibition of bacterial growth. One such examples is the development of new tools for odontology, where AgNPs have been added to customary compounds for dental implants (e.g., polydopamine and titanium) to improve biocompatibility and provide for added antibacterial efficacy against *S. mutans*, commonly implicated in the caries disease (Choi et al., 2019). Green synthesized AgNPs by *Epigallocatechin*

gallate (green tea extract) as reducing and chitosan as capping agent decreased the MIC and MBC against *S. mutans* (Yin et al., 2019). What is more, these AgNPs induced lower amounts of lactic acid and polysaccharides in the biofilm, thus enhancing the protective action of the nanoparticle extracts. The synergistic effect of the bio-extracts and AgNPs may be associated to the bactericide activity of the green tea polyphenols and the large surface area of AgNPs which increase the contact with bacteria and facilitate disruption of cell metabolism. In addition, *E. gallate* is able to inhibit the *S. mutans* glucosyltransferase reducing bacterial adherence and biofilm formation. Another interesting application for AgNPs has been its inclusion in toothpaste formulations with promising antibacterial efficacy against *S. mutans* (Ahmed et al., 2019).

Furthermore, the synergistic effect with other conventional antibiotics makes possible the application of AgNPs as an alternative tool to tackle MDR strains. In fact, AgNPs and conventional antibiotics exert their biocidal action via different mechanisms and, therefore, their combination would prevent the development of added resistance. For example, clindamycin has already been combined with AgNPs resulting in lower MICs in a synergistic effect and rifampicin coupled to AgNP increased the antibiotic effect against methicillin resistant bacteria (Khan et al., 2019a).

Gold Nanoparticles

Gold nanoparticles (AuNPs) have also been employed in different fields of biomedical research due to their ease of synthesis, biocompatibility and low toxicity to higher eukaryotes. They are easier to functionalize with different biological moieties like DNA, mRNA, peptides, etc. than their silver counterparts. Moreover, AuNPs present remarkable optical and photoelectric properties that have demonstrated high potential toward the development of new therapy tools (Amendoeira et al., 2020). The chemical processes for the synthesis of AuNPs are similar to those of AgNPs, where the citrate reduction method is clearly the most used method. Still, in the last years, as for AgNPs, several green synthesis methods with plant or other extract have been proposed (Khan et al., 2019b).

AuNPs have been reported to exhibit antimicrobial activity against a wide range of bacteria and fungus (Tao, 2018). Several mechanisms of action have been highlighted as the basis of their antimicrobial properties, namely: AuNPs may bind to the membrane of bacteria, modify the membrane's potential, decrease intracellular ATP levels, disturb intracellular trafficking, aggregate together with proteins and disturb the assembly of tRNA to the ribosome (Cui et al., 2012). Perhaps, the main aspect related to antimicrobial efficacy relates to nanoparticle dispersion and the AuNPs' surface roughness that could interact with the bacteria membrane (Lima et al., 2013). AuNPs have also been proposed as drug carriers, conveyors of gene therapy and photothermal therapy (Li et al., 2019; Masri et al., 2019b).

The use of AuNPs as drug delivery systems for traditional antibiotics has made possible to administrate drugs more effectively and uniformly distributed toward the target tissue, improving the efficacy and biocompatibility of antibiotic-conjugated AuNPs. The surface of AuNPs may be easily

functionalized with small ligands harboring carboxylic acid, hydroxyl, or amine functional groups that can then be used to conjugate antimicrobials (Lee et al., 2017). The so assemble nanoformulation improves solubility of non-water-soluble drugs and allows for controlled and localized release of the antibiotic, for example with an external stimulus (Canaparo et al., 2019). For example, a formulation of imipenem and meropenem on AuNPs increased the antibacterial effect against carbapenems resistant Gram-negative bacteria, like *Klebsiella pneumoniae*, *Proteus mirabilis* and *Acinetobacter baumannii* isolated from human patients, and decreased the MIC while potentiating the effect in antibiotic kill test (Shaker and Shaaban, 2017). Moreover, these studies showed a size-dependent efficacy of the drug system, with optimal efficacy for 35 nm nanoparticles. Another advantage antibiotic delivery system mediated by AuNPs is the drug-controlled release, where AuNPs loaded with Amphotericin B showed an increase of biocidal efficiency of 78%, with less cytotoxicity and hemolytic toxicity to the host when compared to the antibiotic alone (Kumar et al., 2019).

When AuNPs are irradiated by light with an appropriate wavelength, they tend to convert the received energy into heat, thus resulting in a hyperthermal effect capable to induce damage to the membrane structure (Kirui et al., 2019; Amendoeira et al., 2020). The antimicrobial effect through the use of photothermally active nanomaterials may become an interesting tool against antibiotic resistance. For example, near-infrared (NIR) radiation is useful to promote hyperthermy based on AuNPs, effective against *S. aureus* and *E. coli* after 10 min of irradiation at 808 nm (Alhmoud et al., 2017). NIR phototermy using AuNPs has been also used as an efficient technique to eliminate a broader range of microorganism with improved antibiofilm activity. In fact this approach was demonstrated effective at damaging the cell wall of streptococci, such as *S. mutans*, *S. sobrinus*, *Streptococcus oralis* and *S. salivarius* (Castillo-Martínez et al., 2015).

The combination of these two metals, Silver and Gold, in alloy nanoparticles has also been proposed as suitable nanoagent against microbes. In fact, such approach combines the improved stability and ease of functionalization provided by gold, with the higher antimicrobial activity of silver, while avoiding some problems associated with the aggregation and toxicity to the host (Dos Santos et al., 2012). The positive results of this association have been proposed in a system where gold-silver alloy "nanoflowers" decreased the MIC against *E. coli* three-fold when compared to AgNP alone (Yan et al., 2018). Gold-silver alloy nanoparticles have shown their potential to eradicate biofilm and reduce the MICs against Gram-positive and Gram-negative bacteria, which could then be used to circumvent drug resistance (Ramasamy et al., 2016). Kyaw et al. (2017) showed that submitting triangular AuNPs coated by silver to laser irradiation, induced a change of shape to spherical and increased the antibacterial activity.

Other Metallic Nanoparticles

Nanoparticles employing iron (Fe) have been applied due to their antimicrobial properties, which has been associated with the generation of ROS (Arakha et al., 2015). The most used is iron oxide nanoparticles which provide good efficiency in a wide range spectrum mediated by the damage in different structures like proteins or DNA (Saqib et al., 2019). Most of these iron nanoparticles present some magnetic properties that may be used for a range of applications, from diagnostics to therapeutics (Rodrigues et al., 2019). Magnetic nanoparticles have been shown to interfere with the thiol groups at the respiratory base of bacteria and, thus, assisting in disrupting effective metabolism, resulting in biocidal activity against some drug resistant bacteria (Madubuonu et al., 2019). Ion NPs have also been used as delivery vehicles for antibiotics, such as chlorhexidine and erythromycin against *S. mutans* (Vieira et al., 2019).

Copper nanoparticles have also been used as antimicrobial against a wide range of microorganisms including bacteria, fungi, and even algae (Hou et al., 2018; Sardella et al., 2018). As for AgNPs, the size is related to CuNP activity due to the dramatic increase of the surface/volume ratio, which promote the generation of ROS that trigger cell damage according, such as oxidation of proteins, cleavage of DNA/RNA molecules or lipid peroxidation in membranes (Shaikh et al., 2019). Usually, CuNPs are combined within polymers or functionalized in core-shell structures to provide stability and control possible ion leakage (Anyaogu et al., 2008). For example, CuNPs coated with chitosan showed an antibacterial effect comparable to that of traditional oral antimicrobial agents (chlorhexidine and cetylpyridinium chloride) against *S. mutans* (Covarrubias et al., 2018). In another example, the combination of AgNPs and CuNPs was shown to have a preventive and therapeutic effect in mastitis caused by *S. agalactiae* (Kalińska et al., 2019).

Some authors have studied the antibacterial activity of zinc NPs (ZnNPs) against *Streptococcus mitis*, where the biocidal action was associated to ROS induction identified via the increase of superoxide dismutase activity (SOD) (Khan et al., 2016). Moreover, ZnNPs showed the capability to inhibit the formation of biofilm by *S. mitis* in a dose dependent manner, corroborated the evaluation of bapA1 gene expression, which is associated to generation of the biofilm. In another study, ZnNPs showed the capability to inhibit *S. sobrinus* biofilm formation (Aydin Sevinç and Hanley, 2010).

CONCLUDING REMARKS

The progressive emergence of resistance to conventional antibiotics is reducing the ability to control infectious diseases and, particularly, those caused by pyogenic streptococci. To combat this public health threat, several alternative strategies have been proposed, and the promising efficacy *in vitro* of some of these antibacterial approaches has been the focus of attention.

Despite the progress achieved to date, most alternative approaches are of narrow spectrum unlike the broad-spectrum of conventional antibiotics. However, the combined action of one of these alternative approaches with traditional antibiotics may increase the success rate of therapeutics once that most new strategies attenuate bacterial pathogenesis allowing bacteria to be eliminated by antibiotics and action of the immune system. Moreover, combination therapies may decrease the selective

pressure for resistance to antibiotics, and consequently to reduce the rate of emergence of resistance.

Bacteriocins are considered as a hopeful therapeutic alternative caused by its proven efficacy and chemical structural and functional diversity. Nonetheless, the broad use of bacteriocin can also confer threatening for its usage on a large scale, namely, the possible resistance to bacteriocins could limit their future way. Bacteriophages allow the development of specific therapies, phage-derived enzymes can be used as a substitute for conventional antibiotics, for example, phage-derived lysins that degrade peptidoglycan can be considered as an alternative to β-lactam antibiotics. However, the advancement of new laws that regulate the use of Bacteriophages and phage-derived enzymes is necessary. The application of nanomaterials may provide for new therapy tools to assist in tackling the traditional mechanisms of resistance. Still, there is plenty of work ahead to facilitate the translation to the clinics, namely toward better characterization of these materials, the capability to effectively scale-up for widespread use, and clarification of toxicity aspect, which altogether pave the way for robust assessment in clinical trials. Nowadays, the cost associated with the development of nanotechnology platforms is high and, consequently, the use of more conventional therapies are still preferred.

The most significant concern safety of alternative therapies is the gap of understanding of interaction with the human host. Thus, evaluate the impacts of alternative therapies on the host is essential for future widespread application. Future studies must investigate *in vivo* efficacy of combination therapy to assess their potential, impact of the host, and evolution of bacterial resistance.

AUTHOR CONTRIBUTIONS

CA-B and LR-G: revision of literature and drafting the manuscript. AF and PB: revision of literature and drafting and editing the final manuscript. All authors contributed to the article and approved the submitted version.

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In vitro Synergistic Activity of Antimicrobial Combinations Against *bla*~KPC~ and *bla*~NDM~-Producing *Enterobacterales* With *bla*~IMP~ or mcr Genes

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Carbapenemase-producing *Enterobacterales* have become a severe public health concern because of their rapidly transmissible resistance elements and limited treatment options. The most effective antimicrobial combinations against carbapenemase-producing *Enterobacterales* are currently unclear. Here, we aimed to assess the therapeutic effects of seven antimicrobial combinations (colistin-meropenem, colistin-tigecycline, colistin-rifampicin, colistin-erythromycin, meropenem-tigecycline, meropenem-rifampicin, and meropenem-tigecycline-colistin) against twenty-four carbapenem-producing *Enterobacterales* (producing *bla*~KPC~, *bla*~NDM~, coexisting *bla*~NDM~ and *bla*~IMP~, and coexisting *mcr*-1/8/9 and *bla*~NDM~ genes) and one carbapenem-susceptible *Enterobacterales* using the checkerboard assay, time-kill curves, and scanning electron microscopy. None of the combinations were antagonistic. The combination of colistin-rifampicin showed the highest synergistic effect of 76% (19/25), followed by colistin-erythromycin at 60% (15/25), meropenem-rifampicin at 24% (6/25), colistin-meropenem at 20% (5/25), colistin-tigecycline at 20% (5/25), and meropenem-tigecycline at 4% (1/25). The triple antimicrobial combinations of meropenem-tigecycline-colistin had a synergistic effect of 100%. Most double antimicrobial combinations were ineffective on isolates with coexisting *bla*~NDM~ and *bla*~IMP~ genes. Meropenem with tigecycline showed no synergistic effect on isolates that produced different carbapenemase genes and were highly resistant to meropenem (92% meropenem MIC $\geq$ 16 mg/mL). Colistin-tigecycline showed no synergistic effect on *Escherichia coli* producing *bla*~NDM−1~ and *Serratia marcescens*. Time-kill curves showed that antimicrobial combinations achieved an eradication effect ($\geq$ 3 log~10~ decreases in colony counts) within 24 h without regrowth, based on 1 × MIC of each drug. The synergistic mechanism of colistin-rifampicin may involve the colistin-mediated disruption of bacterial membranes, leading to severe alterations in their permeability, then causes more rifampicin to enter the cell and induces cell death. In conclusion, the antimicrobial combinations evaluated in this study may facilitate the successful treatment of patients infected with carbapenemase-producing pathogens.

Keywords: Carbapenemase-producing *Enterobacterales*, *in vitro* synergistic activity, triple antimicrobial combinations, double antimicrobial combinations, highly resistant isolates

INTRODUCTION

During the last decade, carbapenemase-producing *Enterobacterales* (CPE) have gradually become the main pathogen responsible for significant hospital-acquired infections. Because of limited therapeutic options, infections caused by these "super bacteria" are associated with high mortality rates (Logan and Weinstein, 2017). In 2017, the World Health Organization's priority pathogens list indicated that development of novel antibiotics to treat carbapenem-resistant *Enterobacterales* (CRE) was urgently required (Shrivastava et al., 2018). However, in the current post-antibiotic era, novel antibiotics for treating CRE infections are unavailable owing to the lengthy process of drug discovery and low success rate, which has become a serious concern over the past decade (Luepke et al., 2017). Drug-resistant pathogens are resistant to the most frequently used antibiotics and second-line drugs, resulting in an increased burden of infectious diseases (WHO Pathogens Priority List Working Group, 2018). Thus, antimicrobial combinations may offer an alternative for treating CRE pathogens that are resistant to most available therapies.

Among the many mechanisms that mediate CRE resistance, carbapenemase production is the most common (Nordmann et al., 2011). A series of carbapenemases have been identified in *Enterobacterales*. Three classes of β-lactamases often exist in carbapenem-resistant *Enterobacterales*: Ambler classes A, B, and D. Class A and D β-lactamases have serine-based hydrolytic activities, and class B consists of metallo-β-lactamases with zinc in their active site (Merie Queenan and Bush, 2007). The β-lactamase inhibitors currently available for clinical use consist only of serine inhibitors. For instance, ceftazidime-avibactam works against CRE strains producing *Klebsiella pneumoniae* carbapenemases (KPCs) and OXA-48 but shows no activity against class B β-lactamases. So far, there are no alternative drugs to combat the production of metallo-β-lactamase isolates (Shields et al., 2018).

Combination therapy is a method wherein two or more active antibiotics are used together. This method reduces the frequency of drug resistance and minimizes the dosage of toxic drugs, achieving more significant effects than monotherapy in biochemical activity (Fitzgerald et al., 2006). The Chinese XDR Consensus Working Group (Guan et al., 2016) and most retrospective studies (Nabarro and Veeraraghavan, 2015; Bassetti et al., 2016; Trecarichi and Tumbarello, 2017; Wang et al., 2019) have reported that combination therapy is more effective than monotherapy. However, the advantages of combination therapy remain debatable because different infectious pathogens produce different carbapenemase genes and have different levels of resistance. Investigations focusing on antibiotic combinations that are most effective for treating infections caused by these isolates are limited.

In this study, we explored the synergistic effect of seven antimicrobial combinations against 24 CPE (producing *bla*$_{KPC}$, *bla*$_{NDM}$, both *bla*$_{NDM}$ and *bla*$_{IMP}$, and both *mcr*-1/8/9 and *bla*$_{NDM}$ genes) and one carbapenem-susceptible *Enterobacterales* (CSE) *in vitro*. In addition, the antibacterial synergistic mechanism of colistin with rifampicin was tested using scanning electron microscopy (SEM). The aim of this study was to study the most effective antimicrobial combinations against carbapenemase-producing *Enterobacterales in vitro* activity.

MATERIALS AND METHODS

Microbiological Characteristics of CRE and CSE Isolates

Twenty-five clinical isolates were retrospectively collected from 16 tertiary hospitals in China in 2013–2018 years. The isolates were sent to Peking University People's Hospital for reappraisal of both resistance mechanisms and antimicrobial susceptibility testing (AST). The isolates were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Daltonics Inc., Billerica, MA, United States) or a Vitek 2 compact system (BioMérieux Vitek Inc., Hazelwood, MO, United States). Minimum inhibitory concentrations (MICs) were determined by broth microdilution methods according to the CLSI document M100-S30.[1] For all CPE isolates, polymerase chain reaction (PCR) was used to detect carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{IMP}$) as previously described (Yigit et al., 2008; Xiaojuan et al., 2014; Khodadadian et al., 2018). The colistin-resistant genes *mcr*-1, *mcr*-8, and *mcr*-9 were also detected by PCR as previously described (Quan et al., 2017; Wang et al., 2018; Yuan et al., 2019). Multilocus sequence typing (MLST) was confirmed according to the Pasteur Institute MLST website[2] for *K. pneumoniae* and the MLST websites for *Escherichia coli*[3] and *Enterobacter cloacae*.[4]

Synergy Testing by Checkerboard Assay

The synergy of double or triple antimicrobial combinations were determined using the standard broth microdilution checkerboard assay as described previously (Berenbaum, 1978; Yoon et al., 2004). In brief, the MICs of antimicrobials were determined before the experiment. Ninety-six-well microtiter plates were arranged with increasing concentrations of one drug, ranging from 0.125 to 8 × MIC on the *x*-axis and increasing concentrations of the other drug ranging from 0.125 to 8 × MIC on the *y*-axis. When using triple antimicrobial combinations, fixed concentrations of the drugs were added into 96-well microtiter plates. The final inoculum in each well was approximately 5 × 10^5 CFU/mL. The 96-well microtiter plates were incubated at 37°C for 24 h, and turbidity was observed by the naked eye to determine growth. The effects of the antimicrobial combinations were defined according to the fractional inhibitory concentration index (FICI). FICI = (MIC drug A/MIC drug A plus drug B) + (MIC drug B/MIC drug A plus drug B), FICI ≤ 0.5, synergism; 0.5 < FICI ≤ 4, no interaction or FICI > 4, antagonistic (Odds, 2003). With the triple antimicrobial combination, FICI < 1, synergistic, FICI = 1, additive, or FICI > 1, antagonistic (Berenbaum, 1978).

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[1] http://www.clsi.org
[2] http://bigsdb.pasteur.fr/klebsiella/klebsiella.html
[3] http://mlst.warwick.ac.uk/mlst/dbs/Ecoli
[4] https://pubmlst.org/ecloacae/

Static Time-Kill Assay

A static time-kill assay was conducted for four isolates according to the previously described methodology (Lin et al., 2018). Two *Klebsiella pneumoniae* (bla_{KPC-2}, bla_{NDM-1}), 1 *E. coli* (bla_{NDM-1}), and 1 *Serratia marcescens* were selected to examine the bactericidal effects. The double and triple antimicrobial combinations colistin-meropenem, colistin-rifampicin, colistin-tigecycline, colistin-erythromycin, and colistin-meropenem-tigecycline were tested. Bacteria (1×10^6 CFU/mL) were inoculated in Mueller-Hinton broth containing antibiotics with continuous shaking overnight at $35°C$ and 200 rpm in an atmospheric environment. One hundred microliter samples were drawn and then serially diluted at 0, 4, 8, 16, and 24 h, and 50 µL aliquots were smeared on Mueller-Hinton agar plates. After incubating the plates overnight at $35°C$, the colonies were counted. Synergy was defined as a decrease of ≥ 2 log_{10} CFU/mL between the combination and the most efficient agent alone at 24 h. Bactericidal activity was defined as ≥ 3 log_{10} CFU/mL reduction in cell numbers compared to the initial inoculum after 24 h (Doern, 2014).

Scanning Electron Microscopy (SEM)

The colistin-sensitive isolate SF-18-09 was selected to explore the synergistic mechanism of colistin with rifampicin on cellular morphology using SEM as per a previously described method (Zhang et al., 2019). Bacteria at the mid-exponential growth phase (1×10^6 CFU/mL) were added to the final drug concentration according to the checkerboard results, and a no-drug group was used as a control. The cells were incubated for 4 h, as described in the static time-kill assay method. After incubation, samples were transferred to 15 mL polypropylene tubes (Corning, United States) and centrifuged at $10,000 \times g$ for 3 min. The supernatants were discarded, and the bacterial pellets were resuspended and washed in 1 mL of 2.5% glutaraldehyde in phosphate-buffered saline (PBS). The tubes were fixed overnight at $4°C$. Once fixed, the tubes were centrifuged again at $10,000 \times g$ for 3 min, and the supernatants were removed. Bacterial pellets were resuspended in 1 mL PBS and then observed using a scanning electron microscope (Hitachi SU8020).

Statistical Analysis

Statistical analysis was performed with the software GraphPad Prism version 8.

Ethical Approval

This study was approved by the research ethics board at Peking University People's Hospital. As this study was retrospective and participants were anonymized, informed consent was not required.

RESULTS

Microbiological Characteristics of CRE Isolates

Genotypic and phenotypic characteristics of CRE and CSE isolates used in this study are displayed in **Table 1**, including 11 *K. pneumoniae* (6 bla_{KPC}, 3 bla_{NDM}, 1 coexisting *mcr*-8 and bla_{NDM}, and 1 coexisting bla_{NDM} and bla_{IMP}), 6 *E. coli* (4 coexisting bla_{NDM} and *mcr*-1, 2 bla_{NDM}), 5 *E. cloacae* (2 bla_{NDM}, 1 coexisting bla_{NDM} and *mcr*-9, 1 coexisting bla_{NDM} and bla_{IMP}, and 1 non-carbapenemase producer), 2 *K. oxytoca* (both coexisting bla_{NDM} and bla_{IMP}), and 1 *S. marcescens*. The antimicrobials had the following MICs (µg/mL) against all isolates: rifampicin, 8–128; colistin, 0.125–256; meropenem, 0.125–256 (most isolates (23/25) $\geq$ 16); tigecycline, 0.064–8; and erythromycin, 64–256.

In vitro Evaluation of Synergy Using the Checkerboard Method

We used the broth microdilution checkerboard method to test the following seven antimicrobial combinations: colistin-meropenem, colistin-tigecycline, colistin-rifampicin, colistin-erythromycin, meropenem-tigecycline, meropenem-rifampicin, and colistin-meropenem-tigecycline.

The double antimicrobial combinations of colistin-rifampicin had the highest synergistic effect at 76% (19/25), followed by colistin-erythromycin at 60% (15/25), meropenem-rifampicin at 24% (6/25), colistin-meropenem at 20% (5/25), colistin-tigecycline at 20% (5/25), and meropenem-tigecycline at 4% (1/25). The triple antimicrobial combinations of meropenem-tigecycline-colistin showed a synergistic effect of 100% (16/16). Colistin-tigecycline was ineffective on *E. coli* (**Table 2**).

For CPE isolates, most double antimicrobial combinations were ineffective on the isolates with coexisting bla_{NDM} and bla_{IMP} genes, including colistin-rifampicin, colistin-meropenem, colistin-tigecycline, and meropenem-tigecycline (**Table 3**). Meropenem-tigecycline had no synergistic effect on CPE with highly resistant to meropenem. In contrast to colistin-rifampicin, meropenem-rifampicin demonstrated the greatest potential synergistic effect on isolates with coexisting bla_{NDM} and bla_{IMP} genes, with a synergistic effect of 75% (3/4). Colistin-meropenem also had a synergistic effect of 14.3% (1/7) against bla_{KPC}-producing isolates and 37.5% (3/8) against bla_{NDM}-producing isolates. Colistin with tigecycline had no synergistic effect on bla_{NDM-1}-producing *E. coli* and *S. marcescens*.

Time-Kill Assay of the Antimicrobial Combinations

Time-kill curves of colistin, tigecycline, meropenem, erythromycin, and rifampicin monotherapy or combination therapy against the two *K. pneumoniae* (SF-18-09, bla_{KPC-2}, C2772, bla_{NDM-1}), one *E. coli* (C297, bla_{NDM-1}), and one *S. marcescens* (C261) are shown in **Figure 1**. The data represent the changes in bacterial density from an initial inoculum. The antimicrobial combinations that demonstrated synergy via the checkerboard assay were evaluated using the time-kill assay. According to the checkerboard synergistic drug concentration, antimicrobial monotherapy showed no bactericidal effect on all isolates within 24 h. Conversely, the majority of antimicrobial combinations therapies resulted in an early synergistic effect (≥ 2 log_{10} decrease in colony counts) within 4 h. However, the bacteria showed regrowth over 4 h and had the same growth tendency

TABLE 1 | The characteristics of clinical CRE strains in this study.

Number	Bacteria	β-lactamase		COL-R	MLST	COL	MEM	TGC	RIF	ERY
		Class-A	Class-B							
SF-18-03	*kpn*	NDM-9				0.125	64	0.125	128	64
SF-18-04	*kpn*	KPC-2	–	–	ST11	0.25	128	0.25	16	>256
SF-18-09	*kpn*	KPC-2	–	–	ST11	0.25	128	0.25	16	>256
SF-18-33	*kpn*	KPC-2	–	–	ST11	0.25	128	1	16	>256
SF-18-121	*kpn*	KPC-2	–	–	ST11	0.25	16	0.25	16	>256
C3469	*kpn*	KPC-2	–	–	ST11	8	256	4	16	>256
C3497	*kpn*	KPC-2	–	–	ST11	16	256	4	16	>256
SF-18-153	*kpn*	–	NDM-9	–	ST3387	0.25	256	8	32	>256
SF-18-03	*kpn*	–	NDM-9	–	ST520	0.25	64	0.25	128	64
C1376	*eco*	–	NDM-1	–	ST167	2	64	0.125	16	64
C2772	*kpn*	–	NDM-1	–	ST656	8	128	0.5	16	64
C297	*eco*	–	NDM-1	–	ST469	>256	0.125	0.5	128	256
C2550	*ecl*	–	NDM-5	–	ST25	16	16	0.25	16	>256
C3593	*ecl*	–	NDM-5	–	ST1059	2	256	0.25	16	128
C2413	*ecl*		NDM-5 + IMP-4	–	ST256	0.25	128	0.5	16	256
C2896	*kpn*		NDM-5 + IMP-4	–	ST711	0.25	64	8	128	>256
C3012	*kox*	–	NDM-5 + IMP-4	–	–	0.064	64	0.125	128	>256
C2997	*kox*	–	NDM-5 + IMP-4	–	–	0.25	32	0.25	128	>256
C599	*eco*	–	NDM-5	mcr-1	ST10	4	128	1	128	>256
C613	*eco*	–	NDM-5	mcr-1	ST10	4	64	1	128	>256
C1858	*eco*	–	NDM-5	mcr-1	ST10	4	64	0.25	8	>256
C1930	*eco*	–	NDM-5	mcr-1	ST617	4	128	0.125	8	>256
C185	*kpn*	–	NDM-1	mcr-8	ST37	16	32	8	128	>256
SF-18-202	*ecl*	–	NDM-1	mcr-9	ST55	>256	64	0.25	8	>256
SF-18-28	*ecl*	–	–	–	ST365	64	0.032	0.25	16	256
C261	*sma*	–	–	–	–	>256	128	4	32	256

COL, colistin; COL-R, colistin resistance gene; MLST, Multilocus sequence typing; TGC, tigecycline; MEM, meropenem; RIF, rifampicin; ERY, Erythromycin; MIC, minimum inhibitory concentration; CRE, Carbapenem-resistant Enterobacterales; kpn, Klebsiella pneumonia; ecl, Enterobacter cloacae; eco, Escherichia coli; sma, Serratia marcescens; kox, Klebsiella oxytoca; KPC, K. pneumoniae carbapenemase; NDM, New Delhi metallo-β-lactamase; IMP, imipenemase; mcr, mobile colistin resistance.

TABLE 2 | Checkerboard results of double and triple antimicrobial combinations for 25 clinical CRE strains.

	No. of synergy isolates/no. of isolates tested (%)					
	By organism					
Antimicrobial combination	*K. pneumoniae* (*n* = 11)	*E. coli* (*n* = 6)	*E. cloacae* (*n* = 5)	*K. oxytoca* (*n* = 2)	*S. marcescens* (*n* = 1)	Total (*n* = 25)
MEM + TGC	0/11 (0%)	0/6 (0%)	0/5 (0%)	0/2 (0%)	1/1 (100%)	1/25 (4.0%)
MEM + COL	2/11 (18.2%)	0/6 (0%)	2/5 (40.0%)	0/2 (0%)	1/1 (100%)	5/25 (20.0%)
COL + TGC	4/11 (36.4%)	0/6 (0%)	1/5 (20.0%)	0/2 (0%)	0/1 (0%)	5/25 (20.0%)
COL + ERY	7/11 (63.6%)	3/6 (50.0%)	3/5 (60.0%)	1/2 (50.0%)	1/1 (100%)	15/25 (60.0%)
COL + RIF	9/11 (81.8%)	5/6 (83.3%)	4/5 (80.0%)	0/2 (0%)	1/1 (100%)	19/25 (76.0%)
MEM + RIF	2/11 (18.2%)	1/6 (16.7%)	1/5 (20.0%)	2/2 (100%)	0/1 (0%)	6/25 (24.0%)
MEM + COL + TGC	6/6 (100%)	6/6 (100%)	2/2 (100%)	2/2 (100%)	0/0 (100%)	16/16 (100%)

COL, colistin; TGC, tigecycline; MEM, meropenem; RIF, rifampicin; ERY, erythromycin; CRE, carbapenem-resistant Enterobacterales.

as the control group within 24 h, including those treated with the double (**Figures 1A$_1$,B$_1$,C$_1$,D$_1$**) and triple antimicrobial combinations (**Figure 1D$_1$**). Only the colistin-erythromycin combination showed a bactericidal effect on SF-18-09 within 16 h (**Figure 1B$_1$**).

When using an antimicrobial concentration of $1 \times$ MIC, antimicrobial monotherapy showed no bactericidal effect on all strains within 24 h, whereas the antimicrobial combination therapy achieved an eradication effect (≥ 3 $\log_{10}$ decreases in colony counts) over 24 h without regrowth

TABLE 3 | Checkerboard results of double and triple antimicrobial combinations for 25 clinical CRE strains by CPE.

| | No. of synergy isolates/no. of isolates tested (%) | | | | |
| | By CPE | | | | |
Antimicrobial combinations	KPC (n = 7)	NDM (n = 7)	NDM + mcr (n = 6)	NDM + IMP (n = 4)	Total (n = 24)
MEM + TGC	0/7 (0%)	0/7 (0%)	0/6 (0%)	0/4 (0%)	0/24 (0%)
MEM + COL	1/7 (14.3%)	2/7 (28.6%)	1/6 (16.7%)	0/4 (0%)	4/24 (16.7%)
COL + TG	2/7 (28.6%)	2/7 (28.6%)	1/6 (16.7%)	0/4 (0%)	5/24 (20.8%)
COL + ERY	5/7 (71.4%)	3/7 (42.9%)	4/6 (66.7%)	2/4 (50.0%)	14/24 (58.3%)
COL + RIF	6/7 (85.7%)	6/7 (85.7%)	6/6 (100%)	0/4 (0%)	18/24 (75.0%)
MEM + RIF	0/7 (0%)	1/7 (14.3%)	2/6 (33.3%)	3/4 (75.0%)	6/24 (25.0%)
MEM + COL + TGC	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)	16/16 (100%)

COL, colistin; TGC, tigecycline; MEM, meropenem; RIF, rifampicin; ERY, Erythromycin; KPC, *K. pneumoniae carbapenemase*; NDM, *New Delhi metallo-β-lactamase*; IMP, *integron-encoded metallo-β-lactamase*; mcr, *mobile colistin resistance*; CRE, *carbapenem-resistant Enterobacterales*; CPE, *carbapenemase-producing Enterobacterales*.

(**Figures 1A$_2$,B$_2$,C$_2$,D$_2$**). Colistin-tigecycline only had a bactericidal effect on C2772 (*K. pneumoniae*, bla_{NDM-1}) isolates (**Figure 1D$_2$**) and a synergistic effect on SF-18-09 (*K. pneumoniae*, bla_{KPC-2}) isolates (**Figure 1B$_2$**), and was ineffective against C297 (*E. coli, bla_{NDM}*) (**Figure 1C$_2$**) and C261 (*S. marcescens*) isolates (**Figure 1A$_2$**). In addition, 58.8% (10/17) of the time-kill results were consistent with the checkerboard results.

Impact of Combination Therapy on Cellular Morphology

As the colistin-rifampicin combination showed the best synergistic effect on CPE isolates, we examined their potential synergistic mechanism. The colistin-sensitive isolate SF-18-09 (*K. pneumoniae*, bla_{KPC}) was selected for studying the morphological changes in the bacterial cellular surface using SEM. On treatment with a combination of colistin and rifampicin, the cellular surface showed more micelles and deep craters (**Supplementary Figure 2D**) than in the control group (**Supplementary Figure 2A**). The cellular surface appeared to burst, causing excessive leakage of the cellular contents. In contrast, colistin monotherapy (**Supplementary Figure 2B**) caused only slight asperities and craters on the cellular surface. Rifampicin monotherapy (**Supplementary Figure 2C**) led to the formation of a biofilm layer around the cells, protecting them from being killed.

DISCUSSION

In this study, we assessed the therapeutic effect of seven antimicrobial combinations (colistin-meropenem, colistin-tigecycline, colistin-rifampicin, colistin-erythromycin, meropenem-tigecycline, meropenem-rifampicin, and colistin-meropenem-tigecycline) against 25 clinical isolates producing different resistance genes (bla_{KPC}, bla_{NDM}, coexisting bla_{NDM} and bla_{IMP}, coexisting *mcr-1/8/9* and bla_{NDM}) and preserving highly resistant to meropenem (92% meropenem MIC $\geq$ 16 µg/mL) using a checkerboard assay, time-kill curves, and SEM.

Antimicrobial combination therapy aims to achieve bactericidal effects at sub-MICs of the concerned isolates and is important for extending life and reducing economic burden. Colistin is a polypeptide antibiotic that causes rapid bacterial killing in a concentration-dependent manner. It acts on the Gram-negative bacterial cell wall, leading to rapid changes in the permeability of the cell membrane and ultimately cell death (Newton, 1956; Schindler and Osborn, 1979). There are major concerns regarding the safety of colistin doses and the prevention of heteroresistant phenotypes (Owen et al., 2007).

Our current study yielded several notable findings. First, the double antimicrobial colistin-rifampicin combinations showed the highest synergistic effect against all the isolates tested, but was ineffective against isolates with coexisting bla_{NDM} and bla_{IMP}. Although colistin combined with rifampicin is generally regarded as safe for multidrug-resistant *A. baumannii* infections in clinical settings (Bassetti et al., 2008), it is uncertain whether it can be used to treat infections caused by CPE, the *in vivo* evidence remains insufficient.

Colistin-erythromycin had a suboptimal synergistic effect. The antibacterial spectrum of erythromycin mainly targets Gram-positive cocci, with side effects involving liver toxicity and temporary hearing impairment (Mylonas, 2011). Thus, combining colistin with erythromycin may be a feasible method to alleviate its side effects. Although we identified a significant advantage of combining colistin with erythromycin *in vitro*, there is incomplete information in the literature regarding its possible therapeutic effect on CPE infections, and there is a lack of prospective clinical trials to confirm this effect. The potential mechanism of the combination of colistin and erythromycin may be that colistin increases the entry of erythromycin into the cell, playing an indirect role in its bactericidal activity (Vaara, 1992; Ofek et al., 1994).

The combination of meropenem with tigecycline had no synergistic effect on CPE with highly resistant to meropenem. This result is consistent with a recently published study, which reported that combination with meropenem is becoming less effective against strains with meropenem MIC > 8 µg/mL (Del Bono et al., 2017).
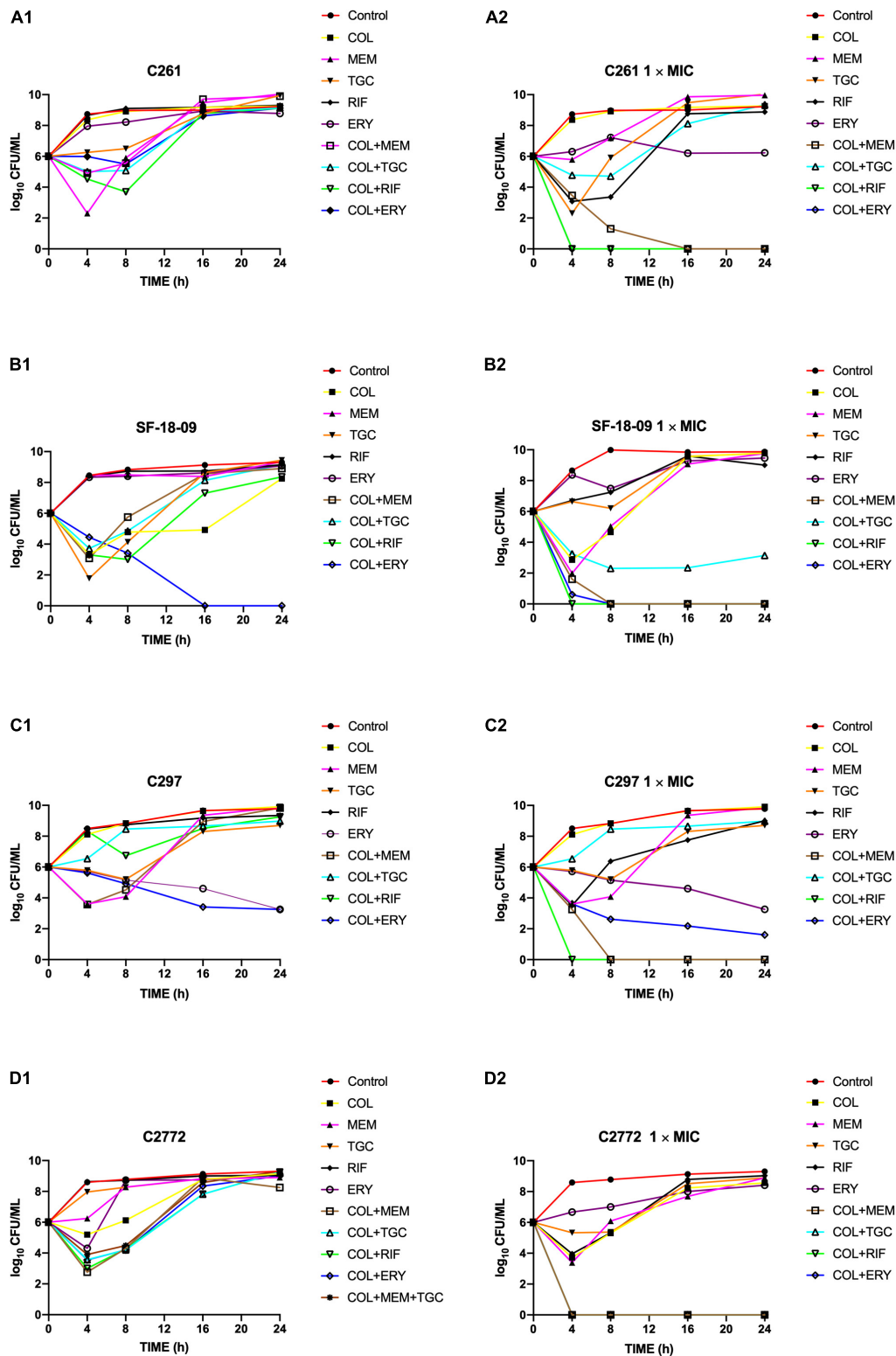
FIGURE 1 | Time-kill curves of colistin alone or in combination with meropenem, tigecycline, rifampicin, and erythromycin against *Serratia marcescens* C261 **(A)**, *Klebsiella pneumoniae* SF-18-09 **(B)**, *Escherichia coli* C297 **(C)**, and *K. pneumoniae* C2772 **(D)**. According to the checkerboard synergistic drug concentration, monotherapy or combination therapy showed no bactericidal effect on clinical carbapenem-resistant *Enterobacterales* (CRE) isolates **(A₁, B₁, C₁, D₁)**. When using antibiotic concentration 1 × MIC, combination therapy achieved an eradication effect (≥ 3 log₁₀ decrease in colony counts) within 24 h without regrowth **(A₂, B₂, C₂, D₂)**.

Triple antimicrobial combinations are being considered as a treatment option against serious CPE infections, and have shown promising results *in vitro* (Diep et al., 2017). In our study, the triple antimicrobial combinations of meropenem-tigecycline-colistin showed a synergistic effect of 100%. This is the first study demonstrating the effectiveness of triple antimicrobial combinations against coexisting carbapenemase gene isolates, and more clinical trials are required to validate their effectiveness.

We also confirmed the checkerboard results using time-kill assays, which provided dynamic measurements of bactericidal activities over time to explore the *in vitro* bactericidal effects. Four strains were selected for this analysis. According to the checkerboard synergistic drug concentration, unsatisfactory bactericidal activity was observed against all strains within 24 h when isolates were treated using either monotherapy or combination therapy, and the same growth tendency was observed as in the control group. However, these results are in contrast with those of another study conducted by Soudeiha et al. (2017), showing that antimicrobial combinations at sub-MIC levels can also prevent bacterial regrowth. The discrepancy between these results may be explained by the use of isolates with different carbapenemase-producing and resistant levels. When using antimicrobial concentrations of $1\times$ MIC, the combination of antimicrobials achieved an eradication effect ($\geq$ 3 $\log_{10}$ decreases in colony counts) by 24 h without regrowth compared with monotherapy, which showed regrowth. The combination of colistin with other antimicrobials also show bactericidal effects on *S. marcescens* strains that are intrinsically non-susceptible to colistin, as several classes of available antibiotics can penetrate the envelope barrier effectively in the presence of colistin (Fajardo et al., 2013). We firstly found that colistin-tigecycline had no synergistic bactericidal effect on bla_{NDM-1}-producing *E. coli* and *S. marcescens.*

SEM was used to observe morphological changes in the bacterial cellular surface. Rifampicin monotherapy resulted in the production of a layer of biofilm formation around cells, protecting them from death. Previous reports have demonstrated that the ability of bacteria to form biofilms may contribute to treatment failure as biofilm-forming bacteria are less susceptible to antibiotics (Donlan and Costerton, 2002). Colistin combined with rifampicin caused more micelles and deep craters than monotherapy, which has been shown to be a precursor of cell death according to the carpet model hypothesis (Ciumac et al., 2019). We also observed structural damage via toroidal pore formation, followed by damage to the bacterial membrane and cell death. This phenomenon strongly supports the notion that the synergistic mechanism of colistin with rifampicin may involve changes in the outer cell membrane permeability encoded by colistin, allowing more rifampicin to enter and kill cells. Another possible mechanism is that the combination of colistin with rifampicin reduces the viability of the cell biofilm at low rifampicin concentrations (Geladari et al., 2019).

In conclusions, we found that the double antimicrobial combinations of colistin with rifampicin had the highest synergistic effect on isolates that produced different carbapenemase genes and were highly resistant to meropenem. However, this combination was ineffective on isolates with coexisting bla_{NDM} and bla_{IMP} genes. The triple antimicrobial combinations of meropenem, tigecycline, and colistin had a synergistic effect of 100%. Colistin with tigecycline had no synergistic effect on bla_{NDM-1}-producing *E. coli* and *S. marcescens*. The limitations of this study include the lack of *in vivo* experiments conducted, in addition to its limited sample sizes. Whether these *in vitro* findings can be applied to a clinical setting needs to be confirmed in further studies, including PK/PD (pharmacokinetics/pharmacodynamics), *in vivo* experiments, and prospective randomized clinical trials. In general, the antimicrobial combinations evaluated in this study may facilitate the successful treatment of patients infected with Carbapenemase-producing *Enterobacterales*.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

HW conceived and designed the study and revised the draft. CZ, QW, LJ, RW, YY, JZ, and SS performed the experiments described in this study. CZ performed the statistical analysis and wrote the draft. All authors approved the final version of the manuscript.

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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.2020.533209/full#supplementary-material

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Nanoparticle-Based Devices in the Control of Antibiotic Resistant Bacteria

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Antimicrobial resistance (AR) is one of the most important public health challenges worldwide as it represents a serious complication that is able to increase the mortality, morbidity, disability, hospital stay and economic burden related to infectious diseases. As such, the spread of AR–pathogens must be considered as an emergency, and interdisciplinary approaches must be undertaken in order to develop not only drugs, but holistic strategies to undermine the epidemic and pathogenic potentials of multi-drug resistant (MDR) pathogens. One of such approaches has focused on the use of antimicrobial nanoparticles (ANPs), as they have demonstrated to possess strong antimicrobial effects on MDR pathogens. On the other hand, the ability of bacteria to develop resistance to such agents is minimal. In this way, ANPs may seem a good choice for the development of new drugs, but there is no certainty about their safety, which may delay its translation to the clinical setting. As MDR pathogens are quickly becoming more prevalent and drug development is slow and expensive, there is an increasing need for the rapid development of new strategies to control such agents. We hereby explore the possibility of designing ANP-based devices such as surgical masks and fabrics, wound dressings, catheters, prostheses, dentifrices, water filters, and nanoparticle-coated metals to exploit the potential of such materials in the combat of MDR pathogens, with a good potential for translation into the clinical setting.

Keywords: gold nanoparticles, silver nanoparticles, graphene nanoparticles, nanoparticle toxicity, nanoparticle modified devices, antibiotic resistant bacteria

INTRODUCTION

Antibiotics have been considered among the most important discoveries in the medical field as they are of great importance to combat infectious diseases, significantly reducing the total morbidity and mortality of such conditions. However, the misuse, and even the use, of such compounds creates antibiotic resistance (AR), which is a bacterium's ability to keep living and/or reproducing in high antibiotic concentrations where other bacteria of the same species become inactive or die. Bacteria can be intrinsically resistant to certain antibiotics but can also develop resistance to antibiotics via genetic mutations. Furthermore, they can also communicate resistance genes (r genes) by

horizontal gene transfer (HGT) to other bacteria, which enhances the spreading potential of this phenomenon (Munita and Arias, 2016). Moreover, bacteria can become resistant to multiple drugs at the same time, being termed multi-drug resistant (MDR) bacteria, or informally "super bugs." Any bacteria can become MDR, but there is a group that has an enhanced ability to develop this condition, and is comprised by *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp., which are collectively known as the "ESKAPE" group of pathogens (Rice, 2008).

Antibiotic resistance-pathogens are a serious problem because they enhance mortality and morbidity rates, increase the risks of medical procedures and medical costs per procedure, prolong illness and convalescence periods, and attack preferentially immunocompromised and hospitalized patients, complicating their conditions (Tanwar et al., 2014), overall summing to a great economic burden worldwide. On the other hand, AR also represents a serious impairment on modern medicine's ability to treat and prevent bacterial infections. As a consequence, AR is currently considered one of the principal threats to global public health by the World Health Organization (WHO; World Health Organization [WHO], 2018) and the Centers of Disease Control (CDC; Center of Disease Control, 2020), and both Organizations have strongly recommended the development of new strategies to cope with the problem.

The community has responded by implementing regulations on antimicrobial use and availability, active surveillance on AR-strains and antibiotic use, educational programs for both the medical practitioners and the general public, improved sanitation, and even banning antibiotic use for growth promotion in animals and improving farm biosecurity. Nonetheless, such political and social measures are yet to produce a full control on the phenomenon. As a consequence, new antimicrobial agents and derivatives, anti-virulence drugs, ecologic and evolutionary management approaches, and even new therapeutic options like those derived from bacteriophages, enzybiotics, and antimicrobial nanoparticles (ANPs) have been undertaken (Roca et al., 2015). Some of these strategies are, apparently, good candidates for the control of the whole phenomenon, but nanoparticles (NPs) are of special interest as they have shown little potential for the development of bacterial resistance against them; despite this, they have not been shown to be entirely safe for their use as drugs. Nonetheless, they may represent a valuable tool to exert ecological control of AR-bacteria, because of their enhanced residual activity. As such, in this review we will examine the antimicrobial properties of NPs and we will discuss their potential to design devices that may be useful in the control of AR-pathogens when used, not as drugs, but as barriers in the clinical, veterinary, farm and sewage settings.

WHERE IS THE ROOT OF THE PROBLEM?

As early as three years after penicillin was introduced into the market and its use was widespread, penicillin-resistant bacteria became a reality and rendered a new challenge for drug developers, whom responded by developing new antibacterial agents. Such increased rate of antibiotic discovery led the period of 1950–1970 to be considered as the "golden age" of antibiotic discovery. Nonetheless, resistance to antibiotics developed shortly after the introduction of every new class of antimicrobial compound (Davies and Davies, 2010; Zaman et al., 2017), regardless of the mode of action of each antibiotic. Although new interesting approaches to antibiotic development have been successfully applied in the experimental setting (Ling et al., 2015; Stokes et al., 2020), worldwide experience with such drugs foresees the development of resistance against these new options, as resistance to any antibiotic class to date has been detected, and even multiple mechanisms of resistance against each type of compound have been described.

In the beginning of the aforementioned "antibiotic era" AR was an uncommon finding, but such phenomenon has spread slowly and steadily all over the world. In 2019 more than 2.8 million AR-infections were detected only in the United States (US), and such infections produced more than 35,000 deaths (Solomon and Oliver, 2014), while in Europe more than 33,000 people die yearly due to AR-infections, and it is thought that the phenomenon will lead to ten million extra human deaths worldwide by 2050 (Abat et al., 2017). Moreover, AR is not an exclusive phenomenon of developed countries as 43% of the deaths due to nosocomial infections in Thailand occur by MDR-pathogens (Lim et al., 2016). The economic burden of this type of infections is also important as only in the US over 20 billion dollars are lost per year, and worldwide economic losses of more than $100 trillion on a yearly basis have also been reported (Munita and Arias, 2016). In such panorama WHO's (World Health Organization [WHO], 2018) and CDC's (Center of Disease Control, 2020) words of caution on the matter are more than justified.

Despite these evidences, the conception of AR as a pandemic is debatable. For instance, a true epidemic represents a rapid increase in the number of cases, and a pandemic is just a global epidemic. As such, pandemics and epidemics represent a sudden impact on the economy and public health, but they do not impact on the long term any of the aforementioned phenomena. On the opposite end, an endemic may occur in high numbers, but its rate is constant or changes slowly and steadily, and may represent a long term impact on both the public health and the economy. As AR has been steadily increasing since the 1940's, causing a long term impact on the worldwide health and economy systems, it can rather be categorized as an endemic (MacIntyre and Bui, 2017). Whether an endemic rate will maintain, increase or decrease is unpredictable, but the sources of any endemic will always stay the same (Bloom and Cadarette, 2019), and this renders a very positive starting point for tackling the whole AR phenomenon, as the roots of an endemic are mostly always known.

In the US alone over 2 million people acquire a nosocomial infection each year, with around 70% of such infections being caused by MDR-strains (Memoli et al., 2008). On the other hand, up to 87% of farm animals have been described to be resistant to amoxicillin and oxytetracyclin, 85% to trimethoprim,

81% to neomycin and 66% to flumequine, among others (van den Bogaard et al., 2001). Moreover, 90% of seawater-originated bacteria are resistant to at least one antibiotic, and up to 20% are resistant to at least five, while antibiotic traces can be detected in 52% of fish samples (Watts et al., 2017). Finally, different r genes can be found on 27.1% of regular sewage bacteria (Parnanen et al., 2019), while the richness and abundance of r genes and MDR bacteria are vastly increased in airplane sewages (Hess et al., 2019), presumably because of HGT among bacteria that are native from different parts of the world.

Following this line of thought, farms, sewages as well as human and veterinary hospitals are critical sources for the AR endemic, so that action in these sectors must be undertaken in order to control such problem. Although social control measures have been applied as means to limit antibiotic usage and accessibility by the general population, such measures are yet to produce the desired effect of lowering the rate of AR dissemination (Uchil et al., 2014). In this way, an interdisciplinary approach must be conceived, and we think that the strategies proposed in such plan must be aimed at the control of resistant microbes in the aforementioned sectors through the use of newer technologies that difficult AR-bacterial spread and development.

Furthermore, AR might be a problem for the end users of antibiotics, nonetheless, the control of the whole phenomenon may not happen at the patient's treatment level because the aforementioned hot points are reservoirs for MDR-bacteria, where these pathogens may persist even after the treatment of infected patients with new effective drugs. Rather, ecological control in the aforementioned areas might be a complementary strategy in order to prevent such infections in the first place, and to reduce the likelihood for the emergence of new AR-bacteria. However, to our notice there are no biotechnological measures or devices in commercial use in order to cope with this awn of the problem. As such, we think that devices with long-lasting antimicrobial properties, such as those that can be fabricated through NP-based coatings, may be of considerable utility for such application.

FIGHTING FIRE WITH FIRE: NANOTECHNOLOGY TO COMBAT ANTIBIOTIC RESISTANCE

Professor Norio Taniguchi from Tokyo Science University introduced the term "Nanotechnology" in the year 1974, and in this word he described precision manufacturing of materials at the nanometer level. Later, Professor Richard P. Feynman, who was a physicist, gave the concept Nanotechnology in his lecture "There's plenty of room at the bottom" (Rai et al., 2009). In general we used the word "nano" to indicate that something has a size of one billionth of a meter or 10^{-9}, with a word that is synonymous to "dwarf". Thus, the term "nanoparticle" refers to clusters of atoms in the size range of 1–100 nm (Rai et al., 2009), and interestingly, some of them possess reduced production costs (Huh and Kwon, 2011).

Thus, it may seem that ANPs are a recent development, but indeed they have been investigated (Lea, 1889), and even

commercially available, for the last 120 years. To our notice, their first commercial application was an antiseptic named "Collargol", which was made of colloidal silver (Fortescue-Brickdale, 1903) that contained silver particles with an average size of 10 nm. Even when the "nano" nomenclature was not used at that time, the increased efficiency of the nano-scaled silver was recognized (Nowack et al., 2011).

Nowadays, many reports have given details about the antimicrobial properties of some NPs, and even activity against AR-bacteria (Andrade et al., 2013). These nanomaterials (NMs) are divided in mainly two groups, according to their chemistry, such being metallic and non-metallic. A vast variety of NPs exist, but the most important in terms of antibacterial properties are: iron oxide (Fe_3O_4NPs), zinc oxide (ZnONPs), copper oxide (CuONPs), titanium dioxide (TiO_2NPs), silver (AgNPs), gold (AuNPs) graphene oxide (GrONPs), and reduced graphene (rGrNPs) NPs (Yousefi et al., 2017).

Along with Alexander Fleming, Paul Ehrlich was one of the pioneers of the so called "antibiotic era." He was a great promoter of the idea that chemical compounds "able to exert their full action exclusively on the parasite harbored within the organism" could be synthesized, leading to the concept of antibiotics as "magic bullets" to kill pathogens exclusively, without damaging the host. Despite antibiotic's success in their specific task, such bullets have attacked bacteria through only one mechanism at a time, which may have favored AR resistance, as altering the bullet's target has been a common bacterial strategy to escape antibiotic's action. On the other hand, the aforementioned NPs do not bind to a specific receptor on the bacterial cell, rather, they have different mechanisms of action, and this characteristic complicates the development of resistance by bacteria, and broadens the spectrum of each kind of NP.

In general, several of the main toxicological effects of NPs occur by direct contact with the bacterial cell surface (Wang et al., 2017), which makes important to understand their properties. Gram-positive bacteria have a thick layer of peptidoglycan and negatively charged teichoic acids (phosphate groups), while Gram-negative bacteria have a thin layer of peptidoglycan associated to a phospholipid outer membrane with lipopolysaccharides that are also negatively charged. Such structural features are important because the primary interaction of metal-based NPs with bacteria is mediated by electrostatic attraction between opposite charges, which leads to a strong bond that triggers their biologically relevant mechanisms of action, which are different for each metal (Guzman et al., 2012). Moreover, structural factors of the NPs influence their antibacterial activity. For example, its size (a smaller size enhances their surface area, which improves their association with cell wall or cell membrane) (Seil and Webster, 2012; Agnihotri et al., 2014), morphology (shapes that augment their surface enhance their function) and dose (the higher the concentration the greater the result) (Breunig et al., 2008).

For the purposes of this article, we chose three "prototypical" kinds of NPs: silver, gold and graphene. The first one being a metallic NP that has a strong standalone action over bacteria, the second one being another metallic NP that lacks such standalone activity, and the last one being a non-metallic type of NP that

has a strong antibacterial activity on its own, as we will discuss the potential applications of these three kinds of NPs in the development of antimicrobial contraptions.

SILVER NANOPARTICLES

Silver has been known as a metal from as early as 4,000 BCE and it was used for making coins, utensils, containers, jewelry, and other objects (Gharpure et al., 2020). Moreover, such element has been used for a long time for the treatment of ulcers (Alexander, 2009), infections, burns and chronic wounds, as well as to make water potable, nonetheless, its true potential in medicine was unknown at that date as this metal was yet to be used in its nanometric size, which enhances its antimicrobial potential. In recent years the AgNPs have been used as disinfectants, but their use as drugs has been limited as AgNPs can produce *agyrosis*, a condition that causes a blue-gray or purple coloration on the skin, mucosal membranes and conjunctiva (Rai et al., 2009).

The AgNPs have also been used in many applications on the physical and chemical sciences, being synthesized in different morphologies like spherical, conical, rod-like wires, etc., but these applications are beyond the scope of this review (Syafiuddin et al., 2017). On the other hand, AgNP uses in biological sciences to combat AR bacteria in products like toothpaste, deodorants, food packaging, cosmetics and water filters are, on the other hand, of great interest for the present discussion (Gharpure et al., 2020).

Different methods for synthesizing AgNPs have been used, and are based on physical, chemical or biological approaches (Song and Kim, 2009). Physical (top-down) and chemical techniques (bottom-up), in general, yield the highest efficiency and production rates, but these methods involve use of toxic and expensive chemicals (Gharpure et al., 2019). For this situation the green synthesis has been proposed as an eco-friendly, unexpensive, reliable, fast, biocompatible, and clean alternative (Gharpure et al., 2020).

Disregarding their synthesis method, AgNPs have been shown to inhibit a wide range of HIV-1 strains, both Gram positive and Gram negative bacteria and parasites such as *Leishmania tropica, Entamoeba histolytica, Cryptosporidium parvum, Giardia lamblia, Fasciola* spp., *Plasmodium* spp., and *Toxoplasma gondii*, whether resistant to antibiotics or not, therefore they are considered to possess a broad spectrum of action (**Table 1**).

The biological effects of AgNPs is dependent on the following mechanisms: (a) the silver NPs binding to the cell wall altering its permeability, which was demonstrated in studies conducted on gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, where the neutralization of the bacterial surface charge altered the cell membrane's permeability (Ramalingam et al., 2016) and in *E. coli*, where scanning and transmission electron microscopy studies demonstrated that these NPs are able to produce pits in the cell walls, where the AgNPs accumulated (Sondi and Salopek-Sondi, 2004); (b) AgNPs are able to bind to membrane transport and respiratory chain proteins, thus interfering with the cell division and ion transport processes, ultimately leading to bacterial death (Ivask et al., 2014); (c) their nanometric size (1–10 nm) allows them to penetrate into

bacteria affecting intracellular process leading to inhibition of transcription, translation and protein synthesis (Rai et al., 2009; Reidy et al., 2013). This mechanism is explained by the release of silver ions which could be generated and introduced by oxidative dissolution of AgNPs in the presence of oxygen (Reidy et al., 2013). Such ions with positive charge have high affinity with the negatively charged sulfhydryl, phosphate and carbonyl groups of the bacterial cell membrane, proteins and DNA bases (Rai et al., 2009; Xiu et al., 2012; Chung et al., 2016) and upon contact lead to their alteration. Also, they are able to impair the transport and release of potassium ions and to block the synthesis of ATP in the bacterial cells (Hsueh et al., 2015). It has been shown that smaller NPs not only have an enhanced reactivity, but can also release more of these ions to an enhanced antimicrobial activity (Jung et al., 2008). Additionally, (d) AgNPs are able to dissipate the proton motive force of bacterial enzymes and transporters, thus causing an accumulation of envelope protein precursors and causing a destabilization of the outer membrane, collapse of its potential and depletion of intracellular ATP levels (Lok et al., 2006). Finally, (e) AgNP and silver ions lead bacteria to generate reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radical (Manikprabhu and Lingappa, 2013), as well as malondialdehyde, protein carbonyl content, and nitric oxide (Gurunathan et al., 2018), which on increased concentrations cause a direct damage to bacterial DNA causing chain breakage, cytosine, adenine and guanine deamination, lipid peroxidation and also block the activation of the enzymatic DNA repair (**Figure 1**).

As a side note, AgNPs also possess anti-HIV activity, by inhibiting the initial phase of fusion and entry of HIV-1 mediated by the gp120-CD4 interaction in a dose-dependent manner. Also AgNPs inhibit the first phase of the replication cycle at the same place where the antiretroviral enfurtivide acts. Moreover, AgNPs also have antiparasite activity, as they enter directly in the protoctist cell membrane through ion channels, transport proteins or by endocytosis, and then they inhibit promastigote proliferation, damage DNA and generate ROS (Khezerlou et al., 2018).

Gold Nanoparticles

Gold has been known to man since the pre-history, and has been used as a coin since 3100 BCE. It is considered as a noble metal due to its inertness towards other atoms and molecules, which makes for its apparent lack of cytotoxicity to human cells and a great overall biocompatibility (Gharpure et al., 2020). Its innate properties have been exploited for a plethora of biomedical applications like targeted drug delivery, biosensors, optical imaging, as well as detection of microorganisms (Gharpure et al., 2020). Many studies have also shown the antimicrobial properties of this type of NPs, especially in many pathogenic species (Dizaj et al., 2014). AuNPs can be fabricated using "top-down" as well as "bottom-up" methods, however, green synthesis has also been favored for its environmental friendliness (Shah et al., 2014).

Gold nanoparticles (AuNPs) are effective against many strains of bacteria and fungi such as *E. coli, P. aeruginosa, Salmonella typhi, Bacillus subtilis, Candida albicans, Candida glabrata,* and some major pathogenic bacteria like *S. aureus* and *K. pneumoniae*

TABLE 1 | Mechanisms of action and spectrum of activity of nanoparticles.

Nanoparticle	Action Mechanism	Spectrum	References
AgNPs	• Alteration of membrane permeability. • Binding to membrane proteins (respiratory chain proteins, transport proteins) and interfering with cell division and ion transport processes. • Inhibition of transcription, translation and protein synthesis. • Generation of ROS causing a direct damage on DNA.	*Leishmania tropica* *Entamoeba histolytica* *Cryptosporidium parvum* *Giardia lamblia* *Fasciola spp.* *Plasmodium spp.* *Toxoplasma gondii* *Escherichia coli* *Pseudomonas aeruginosa*	Ivask et al., 2014; Habimana et al., 2018; Ahmad et al., 2013; Eigler and Hirsch, 2014; Soleymani et al., 2016; Amrollahi-Sharifabadi et al., 2018
AuNPs	• Interfering with protein and ATP synthesis. • Modifiying membrane potential. • Inhibition of H$^+$ transport	*Escherichia coli* *Pseudomonas aeruginosa* *Salmonella typhi* *Bacillus subtilis* *Staphylococcus aureus* *Klebsiella pneumoniae* *Candida albicans* *Candida glabrata*	Khezerlou et al., 2018; Kumar et al., 2019
Graphene	• Disruption of cell membrane by nano-blades. • Trapping of the bacterial membrane. • Destructive extraction of membrane lipids. • Oxidative stress which interfere with bacterial metabolism.	*Escherichia coli* *Staphylococcus aureus* *Pseudmonoas aeruginosa* *Bacillus subtilis* *Enterococcus faecalis* *Staphylococcus epidermidis*	Mu et al., 2016; Barbero et al., 2017; Ahsan et al., 2018; Kang et al., 2019

FIGURE 1 | Mechanisms of action of nanopaticles. AgNPs 1) bind to cell membrane neutralizing its charge, thus altering its permeability and affecting membrane transport and respiratory chain proteins; 2) release silver ions affecting genetic expression and ATP synthesis; 3) and generate ROS affecting DNA and cell membrane. AuNPs 4) inhibit ATP synthase; 5) inhibit tRNA binding to the ribosome; GrNPs 6) entrapment of the bacterial membrane; 7) form nano-blades that cut the cell membrane and destroy DNA; GrONPs 8) destroy the bacterial cell membrane; and 9) ROS-dependent and ROS-independent oxidative stress.

(**Table 1**), including AR-strains. Unlike AgNPs that act alone, AuNPs act better in association with antibiotics, vaccines and antibodies, as they enhance their intracellular transportation, and diffusion, but alone, their antimicrobial properties are mainly dependent on its ability to interfere in protein synthesis by the inhibition of the tRNA's ability to bind the ribosome (Cui et al., 2012), to inhibit ATP-synthesis by the inhibition of ATP-ase and damaging the microbial cell membrane (Nisar et al., 2019), as well

as their ability to modify the membrane potential (Gharpure et al., 2020). Additionally, these NPs seem to disrupt biofilms, although this activity is greatly enhanced when AuNPs are conjugated with proteinase-K (Habimana et al., 2018). On the other hand, their antifungal activity is due to the inhibition of H^+-ATPase at the plasma membrane, inhibiting the H^+ transport chain (Ahmad et al., 2013; **Figure 1**).

Graphene Nanoparticles

Graphene (Gr) is an allotrope of carbon that consists of a single layer of atoms in a two dimensional lattice, it is the basic structural element of other allotropes, including graphite, charcoal, carbon nanotubes and fullerenes (Eigler and Hirsch, 2014). Since its discovery by Prof. Andre Geim and Prof. Kostya Novoselovt in 2004 through micromechanical exfoliation of graphite (Zhang et al., 2012), Gr has been regarded as the strongest material in the world, and its extreme elasticity and conductivity have also been recognized, placing it as a wonder material with many applications in biomedicine for the diagnostic and treatment of diseases (Amrollahi-Sharifabadi et al., 2018). Moreover, Gr can also be synthesized in an oxidized form to yield graphene oxide (GrO), which possesses chemically reactive oxygen groups such as hydroxyl, carboxylic acid, and epoxy which enhance its reactivity (Soleymani et al., 2016).

Because of their two-dimensional structure Gr and GrO have a large specific surface area that enhances their reactivity and gives them some of their interesting antibacterial properties (Hasanzadeh et al., 2016), but also their size, oxidation level, functionalization, or electronic structure are able to modulate their activity (Perreault et al., 2015). Their proposed mechanisms of action involve both physical and chemical activities, where the physical modes of action are the most extensively found in experimental settings, such as direct damage by contact of the bacterial membranes with the sharp edges of graphene and wrapping of the bacteria. On the other hand, the chemical modes involve oxidative stress by ROS generation and charge transfer (Kumar et al., 2019).

For instance, on the physical damaging strategies of both pristine (Tu et al., 2013) and oxidized (Hui et al., 2014) graphene Tu et al. (2013) observed the degradation on the inner and outer cell membranes of *E. coli*, as well as the reduction of the colony's viability in relation to the ability of Gr to penetrate such structures to extract its phospholipid contents. Such damage resemble the formation of pores on the membrane, and it has been further suggested that the density of Gr edges, rather than the size or shape of such structures, is the main factor enhancing its antibacterial ability (Pham et al., 2015). Moreover, GrNPs are able to cause membrane stress by direct contact with the sharp edges of their nanosheets, which disrupts and damages cell membranes, thus leading to the release of intracellular contents in a mechanism of action termed as "nano-blade." Also, GrNPs are able to aggregate in the surroundings of the bacterial cells to trap bacteria in Gr-based cages, isolating them from the nutrients from the environment and thus leading to bacterial cell death (Liu et al., 2011; Zou X. et al., 2016). Both GrNPs and GrONPs are able to produce ROS-dependent and ROS-independent (derived from the oxidation of glutathione) oxidative stress, which interfere with bacterial metabolism and disrupt cellular functions (Liu et al., 2011; Zou X. et al., 2016), while this effect is stronger for GrO (Liu et al., 2011; **Figure 1**). Both materials have exhibited good antibacterial properties against many bacteria, to include *E. coli, S. aureus, P. aeruginosa, B. subtilis, Enterococcus faecalis,* and *S. epidermidis* (Pham et al., 2015; **Table 1**), many of which are AR strains.
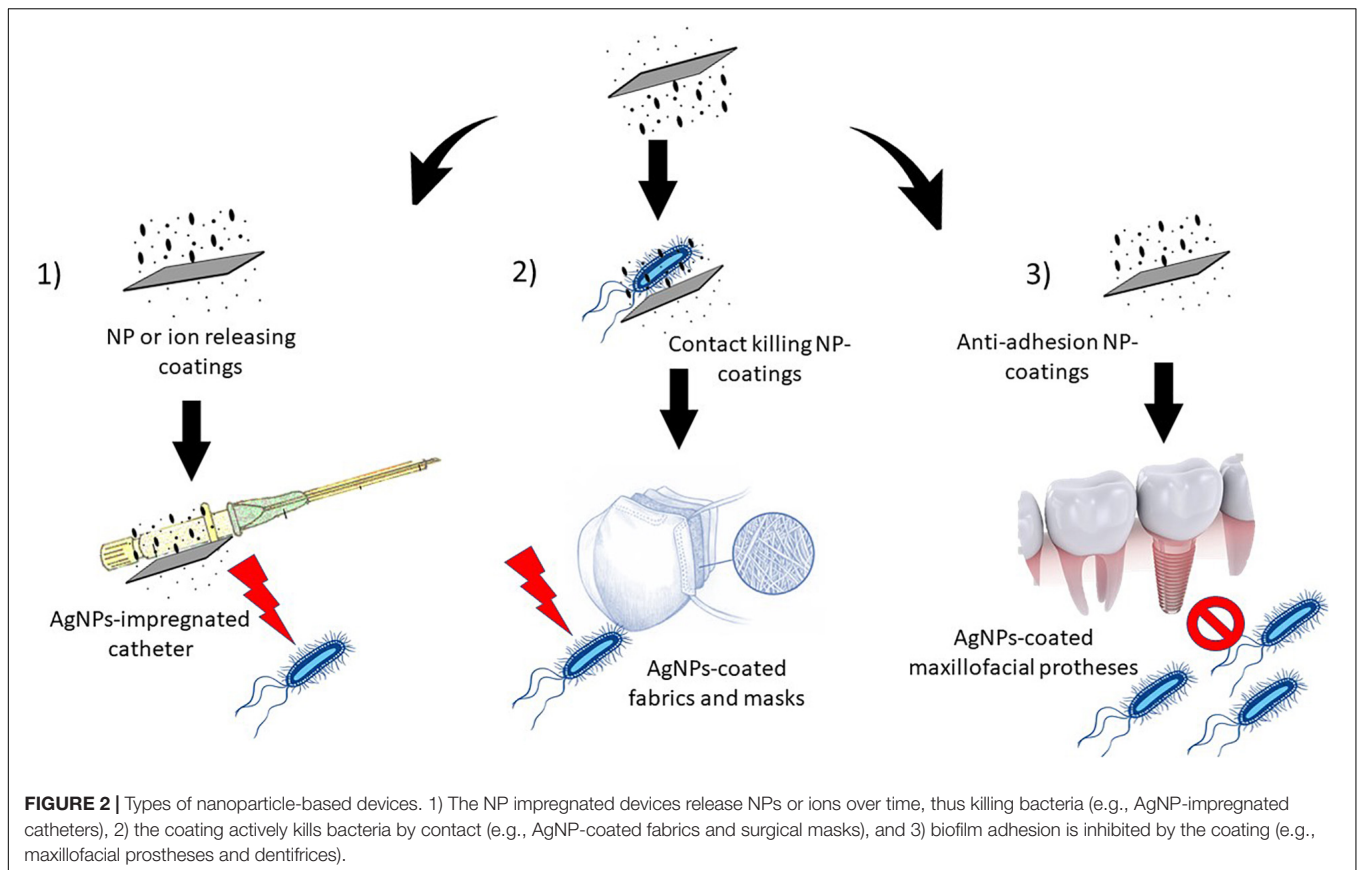
As such, both materials have been used to disperse, stabilize and deliver drugs, like antibiotics, but such materials also exhibit good antimicrobial properties on their own. Such antibacterial activity, coupled with Gr and GrO's flexibility and resistance, has led to the design of antibacterial packaging for food, water treatment devices, wound dressings and disinfectants (Ji et al., 2016).

Summary of Antimicrobial NPs' Mechanisms of Action

As mentioned above, NPs have more than one mode of action over bacteria, which enhances their potential to damage different prokaryotic structures at the same time, thus augmenting their overall antibacterial activity. However, it is not clear if all the reported effects for each NP occur with every bacterial species, nor if all of the antimicrobial mechanisms reported for a particular chemical NP type are the same when the size and shape of the NPs is different. In such panorama, comparative studies to determine what size, shape and chemical type of NPs possess the strongest antibacterial activity, and over what bacterial species do they exert these effects are needed in order to determine the best ways to use these agents. Moreover, this kinds of studies may serve to better assess if resistance to NPs at optimal doses is possible, and may also serve to focus studies about their toxicity in a more efficient way, as these topics have been an intense matter of discussion over the years, finding conflicting results.

Moreover, much of these assays have been carried out *in vitro*, while emerging properties in living systems have not been taking into proper account for the assessment of NPs' antimicrobial activity. For instance, it is known that upon contact with NPs proteins tend to aggregate to the NPs' surface to constitute a structure known as "biomolecular corona," which alters various NP's properties, like targets, toxicity, immune-modulating properties and interaction with cellular structures (Barbero et al., 2017; Ahsan et al., 2018). This phenomenon could also alter the way that NPs interact with bacteria, changing entirely their antimicrobial properties when they are used as drugs. Moreover, antimicrobial NPs do combat intracellular bacteria in co-cultures of bacteria and host cells, but with decreased efficiency in comparison with extracellular bacterial infections (Kang et al., 2019). In such cases the NPs' antibacterial ability was enhanced through conjugation with antibiotics, as NPs worked as vehicles for the antibiotics and synergized with them (Mu et al., 2016). Nonetheless, it is still unknown whether NPs may be able to combat intracellular infections in an *in vivo* setting, in which dose and with what efficiency. Thus, as happens with many emerging nano- and bio-technologies, much research is needed to complete our understanding of NPs' properties, and *in vivo* acquired data is of paramount importance in this case.

FIGURE 2 | Types of nanoparticle-based devices. 1) The NP impregnated devices release NPs or ions over time, thus killing bacteria (e.g., AgNP-impregnated catheters), 2) the coating actively kills bacteria by contact (e.g., AgNP-coated fabrics and surgical masks), and 3) biofilm adhesion is inhibited by the coating (e.g., maxillofacial prostheses and dentifrices).

BACTERIAL RESISTANCE TO NANOPARTICLES: IS IT POSSIBLE?

As NPs act on bacteria by multiple mechanisms at the same time, the development of resistance to them should come from several accumulated mutations as a mean to modify some, or all, of the NPs' bacterial targets. In this way, some authors think that such phenomenon is highly unlikely (Baptista et al., 2018), but others have detected some degree of resistance mainly to metallic NPs, as thoroughly reviewed in (Nino-Martinez et al., 2019). To date, electrostatic repulsion, ion efflux pumps (Yang et al., 2012), production of protective extracellular matrixes (Zhang et al., 2018), mutations (Graves et al., 2015), and biofilm adaptations have been detected among bacteria in order to resist the attack of such NPs (Agnihotri et al., 2014).

Interestingly, the detection of NP-resistance (NPR) has been made in experiments where bacteria have been cultured in mediums with low metallic-NP concentration, but to our knowledge, there is no data about NPR occurring in cultures with the minimum inhibitory concentrations for each type of NP. Moreover, NPR may be countered easily by many strategies, to include the regulation of NP's size as a reduction of their dimensions leads to an increased surface/volume ratio and thus to enhanced antimicrobial activity (Agnihotri et al., 2014), which reduces the possibility of bacterial survival and development of resistance through mutations

(Graves et al., 2015) and development of ion efflux pumps (Yang et al., 2012). Also, agents like simvastatin and pomegranate rind extract have been demonstrated to inhibit the extracellular aggregation of metallic-NPs that occurs through the bacterial production of an extracellular matrix and by flagellin expression (Panacek et al., 2018; Cui et al., 2020). In this way, a correct design of the NP-based therapies or devices may not induce the development of NPR.

To our knowledge, there is no data regarding the development of resistance against GrNPs or GrONPs, but excellent research by Guo and Zhang (2017) and Zou W. et al. (2016) have shown that GrONPs are able to damage r genes-carrying plasmids and lower the abundance of the r genes *sulI* and *intI*, which suggests that resistance to GrONP is unlikely to happen. Furthermore, such investigations suggest the basis of a strategy to halt the HGT of genetic resistance determinants, which may be of use in the dampening of the whole phenomenon of AR.

On the other hand, either bimetallic NP (Gopinath et al., 2016) or GO-Ag nanocomposites (de Moraes et al., 2015) have shown increased antibacterial activity when compared to monometallic or non-composite NPs, but research regarding their ability to halt r genes or about the bacterial ability to develop resistance against such materials is lacking, but their increased antimicrobial abilities appear as promising alternatives to avoid resistance development.

TRANSLATION TO THE CLINICAL SETTING: ARE NANOPARTICLES SAFE TO USE?

The prototypical path to introduce new drugs into the clinical setting requires vast preclinical testing of the candidate products to produce information about their efficacy and safety. Drugs that appear to be safe in animal models and human cell studies, may then be evaluated for their further study in phase 1 clinical trials, where safety and dosage in human patients will be assayed in no more than 100 volunteers, in a period that typically lasts for ≈3 years. If they are found to be safe, further tests of effectiveness, side effects and adverse reactions of the long term will be performed in phase 2 and 3 clinical trials in a period that may last up to ≈13 additional years. Notwithstanding, all new products must prove to be safe in the preclinical setting in order to be approved for phase 1 trials (Lipsky and Sharp, 2001). Accordingly, we and other researchers (Min et al., 2015) think that the translation of some applications of the antimicrobial NPs into the clinical setting may be difficult and/or greatly delayed as there is not much consistency on their safety profile, as contrasting results have been shown on several *in vitro* and *in vivo* models.

For instance, AgNPs have shown good *in vitro* compatibility with human and murine erythrocytes (Hossain et al., 2019), but they do exhibit toxicity to peripheral blood mononuclear cells (PBMCs; Shin et al., 2007). Moreover, *in vivo* studies on zebra fish made by Bao and colleagues show that AgNPs induce pathological changes on growth indices, oxidative/antioxidative status, neural signaling, Na/K-ATPase function, and antioxidant system in the intestine, but not in the liver, with male zebrafish being more sensitive than female (Bao et al., 2020). Despite the absence of liver damage on zebra fish observed by Bao, further studies on liver damage show that although murine healthy livers do not appear to suffer much damage by AgNPs, these NPs appear to enhance ethanol-induced inflammatory damage in such organ (Kermanizadeh et al., 2017). Moreover, although functionalization of AgNPs with either polyvinylpyrrolidone (PVP) or citrate appears to enhance these NP's antimicrobial activity, it enhances their potential to induce cellular toxicity as well as inflammatory and oxidative stress on murine lungs, thus leading to mild pulmonary fibrosis (Wang et al., 2014). Together, these results suggest that AgNPs may possess a certain degree of toxicity that may be impossible to overcome, but on the other hand Rezvani et al. (2019) suggest that AgNPs may be safe on certain limited doses. Nonetheless, a reduction on these NP's doses may lead to NPR (Graves et al., 2015), in such a way that more research is needed in order to best describe a safe and effective way of using AgNPs as drugs.

Although AuNPs are generally thought to be safe, they have been shown to accumulate in the intestine, kidney, liver, spleen, and colon, while also being aggregated in the nucleus of hepatocytes and colonic cells, where they produce DNA damage, with smaller NPs exhibiting more toxicity (Lopez-Chaves et al., 2018). Moreover, such NPs have also been

demonstrated to induce pulmonary inflammation, disregarding their size (Gosens et al., 2010).

Moreover, non-metallic NPs have also shown a certain degree of toxicity as chicken embryos that were injected with diamond, graphite, Gr and GrO NPs into the egg albumin showed decreased survival, but there were no differences on body and organ weight, red blood-cell morphology, blood serum biochemical parameters, and oxidative damage in the surviving embryos in comparison with the placebo group (Kurantowicz et al., 2017). Also, upon inhalation GO nanosheets induce pores in the pulmonary surfactant film, thus altering its ultrastructure and biophysical properties (Hu et al., 2015). Furthermore, in the graphene family of NPs cytotoxicity dependent upon physical destruction of cell components, oxidative stress, DNA damage, inflammatory response, apoptosis, autophagy, and necrosis have been detected in many different models, as thoroughly reviewed in (Ou et al., 2016). Nonetheless, in other *in vivo* studies pathological changes in weight gain, hematological and biochemical parameters have only been detected in rats that received high GrONP-doses (500 mg/kg), but not lower doses (50 or 150 mg/kg) (Amrollahi-Sharifabadi et al., 2018).

Despite the aforementioned evidence, which seems to render antimicrobial NPs mostly as toxic substances, controversy has arisen in regards to this topic, as some authors report a safe profile for NPs while other researchers find cues of their toxicity. This controversy may arise from the fact that NP are not created equally and have different properties. Aside from the dose (the larger the dose, the more toxic it is) (Graham et al., 2017), the size (the smaller the NPs the more toxic that they are) (Pan et al., 2007; Kim et al., 2012), shape (the most surface they have, the more toxic they are) (Wang et al., 2008; Nam and An, 2019), surface charge and chemistry of NPs (Zoroddu et al., 2014) also relate to increased NP toxicity, and may change the NP's absorption properties, accumulation, distribution and elimination, thus complicating the study of their toxicity. On the other hand, surface functionalization of such materials has been proposed as an alternative to improve biocompatibility (Subbiah et al., 2010), but research has shown that in some cases (Wang et al., 2014; Zhang et al., 2016) this strategy enhances toxicity.

Lastly, most of the assessments for NPs' toxicity comes from *in vitro*-performed experiments, where emerging properties of living systems are neglected. In the setting of living beings, at least three physiological phenomena could impact the final toxicological performance of NPs, which are: (a) the fact that cells possess different shapes when they are part of a three dimensional tissue, in comparison to those that are cultivated in two dimension culture plates. This phenomena has only recently been recognized as a factor determining NP uptake and toxicity (Farvadi et al., 2018). Secondly, (b) the formation of the "biomolecular corona" that happens when NPs come in contact with biomolecules, such as proteins (Ahsan et al., 2018) which could alter their toxicological properties significantly. And lastly, (c) the fact that many bacterial infections are intracellular, and that although NPs are able to combat intracellular infections, they do so with decreased efficiency over extracellular colonization (Kang et al., 2019), which may impact the efficiency of the dose.

In this way the study of nanotoxicology becomes increasingly complicated with NP diversity and with the interaction with the host, which may explain the emergence of the aforementioned controversy regarding the safety of NPs for use in medicine. Nonetheless, much research is needed in a standardized manner to accurately assess the toxicity of NPs (Zoroddu et al., 2014; Hofmann-Amtenbrink et al., 2015) in different doses and routes of administration, and then get to know their potential in the clinical setting, which may be of enhanced importance in order to fully explode this promising technology.

Taken together, these data suggest that antimicrobial NPs may not be safe for their use as drugs, or at least, that much knowledge is needed in order to establish the right windows for dosage, administration routes, functionalization strategies and physical properties for their safe use. Thus, this knowledge, or lack of knowledge may halt NPs' approval for clinical use. In such panorama, more research is needed in order to enhance our knowledge about the modulation of NPs' toxicity and activity trough functionalization and regulation of NP's dose, size, shape, and chemistry in order to fully exploit their potential, but also research about alternative uses for NPs that do not involve administration into the human body are critical, as a strategy to exploit their potential as soon as possible and dampen their toxicity at the same time.

One could argue that the last decade has witnessed a rise in nanomedicine, with many kinds of NPs being investigated and applied into the clinics, but the nano-drugs that have been approved for their clinical use, or that are actually being investigated in clinical trials are mainly anti-tumoral agents, supplements, imaging contrasts and agents, drug-delivery vehicles, anesthetics (Anselmo and Mitragotri, 2019), and even as biosensors for the detection of infectious diseases (Colino and Millán, 2018), but little to no advances have been made in regards to ANPs into their clinical application, which reflects the importance of this window of opportunity.

ANTIMICROBIAL NANOPARTICLE-BASED MEDICAL DEVICES: TOWARDS THE ECOLOGIC CONTROL OF MDR BACTERIA

As stated above, many experiments have rendered antimicrobial NPs as good alternatives to fight AR-bacteria, and some types of NPs may even be able to avoid resistance development, but the use of such technology as drug alternatives may be halted due to the numerous evidences about its toxicity. Nonetheless, as promising research shows, antimicrobial NPs may have an alternative niche in the combat of AR pathogens trough NP-based medical devices, where the NPs are not administrated into the body, or have a minimal contact with it, but can be used as barriers in endemic zones for AR to control the spread of AR-bacteria. In this way, such contraptions may exert ecological control of such pathogens while avoiding toxicity to the human body.

As stated in the above sections, hospitals are considered endemic zones for AR-bacteria and AR development, and within a hospital, textile fabrics have been described as important reservoirs and fomites (Neely, 2000; Neely and Maley, 2000; Pilonetto et al., 2004). Moreover, other biomedical devices that come in contact with the patients, like catheters and wound dressings, are also important vectors for the transmission and dissemination of nosocomial bacteria. As such, the prevention of microorganism colonization and consequent biofilm formation in these devices could limit AR-development (Palmieri et al., 2016). Three major types of antimicrobial coatings have been designed to accomplish this task: (i) the ones that work through antibacterial NP release, others that work by (ii) contact-killing, and finally (iii) by halting bacterial adhesion. In the first case, the coating is loaded with a drug that is released over time by diffusion or erosion, and because the release is local and gradual, it limits systemic effects. In the second case, bacteria are directly killed by contact with the coated portion of the device, and in the third, biofilm adhesion becomes impaired (**Figure 2**). In every case, the potential harmful effects of such coatings are limited to the contact area and its near surroundings, so that they appear to be safer than NP-based drug candidates (Palmieri et al., 2016).

Following this line of thought, Duran et al. (2007) developed AgNP-coated cotton fabrics that demonstrated a significant antibacterial activity against *S. aureus*, while Li et al. (2006) developed titanium dioxide and silver-nanoparticle coated surgical masks that killed 100% of both the *E. coli* and *S. aureus* bacteria that were incubated in such masks, without producing skin irritation on their volunteered wearers.

On the other hand, AgNP-coated wound dressings have an enhanced ability to prevent bacterial colonization and biofilm formation at wound sites while promoting tissue regeneration, and thus have been rendered as useful in the treatment of extended wounds and burns (Halstead et al., 2015). Moreover, nanocomposites of silver and graphene with a ratio of 5:1 exhibit stronger antimicrobial and wound healing abilities than other hydrogels, while exhibiting low toxicity as evidenced by an MTT (3-(4,5-Dimethyldiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) assay (Fan et al., 2014), suggesting that nanocomposites may have an enhanced function over simple NPs, perhaps as a function of the addition of two different sets of action mechanisms (**Table 2**).

This technologies could be extrapolated to their use in surgical fields, intensive care unit (ICU) bedding and scrubs for its use in both human and animal hospitals; but also could be used to produce bedding material and protections for pens and cages, or even to produce air filters to control AR-bacteria spreading in farms (**Table 2**).

Furthermore, in the late 90's nano-silver coated catheters were clinically investigated for their potential to reduce hospital acquired bacteremia with disappointing results (Darouiche et al., 1999; Antonelli et al., 2012), nonetheless further preclinical investigations showed that a supercritical carbon dioxide impregnation method, as opposed to coating, showed an increased release of silver ions that could lead to enhanced antibacterial actions (Furno et al., 2004; **Table 2**). In regard to this technology, a thorough investigation on whether the

TABLE 2 | Nanoparticle-based antimicrobial devices: uses and possible future applications.

Devices	Nanomaterial	Advantage	Shown Application	Possible further uses
Cotton fabrics	AgNPs	Significant antibacterial activity against *S. aureus*	Antimicrobial textile fabrics	Surgical uniforms, surgical fields, bedding and scrubs for hospital and veterinary use Bedding materials, pen and cage protections in farms
Surgical masks	Titanium dioxide and silver-nanocomposite	Kills 100% of both, *E. coli* and *S. aureus* without skin irritation	Surgical masks	Air filters coupled to air conditioning devices
Coated wound dressings	AgNPs	Prevent bacterial colonization and biofilm formation at wound sites while promoting tissue regeneration	Treatment of extended wounds and burns	May be used for veterinary applications
Coated catheters	AgNPs	Dampening of catheter-produced bacteremia	Reduction in bacterial colonies	Building components for hemodialyzers, blood oxygenators, and arterial filters, among other biomedical devices. Hoses to transport water into farms. Sewages.
Coated maxillofacial silicone prostheses	AgNPs	Good biocompatibility and antimicrobial actions	Antibacterial maxillofacial prostheses	Sealing materials for building farms and hospitals, sewage joints. Base materials for water bottles and filters
NP-coated polyurethane and polycarbonate	GrO	Good biocompatibility and antimicrobial actions	None	Pipes, toilets, hoses and panels
Dentifrice	Nano-silver fluoride	Effectively kills *S. mutans*, prevents bacterial adhesion to teeth and control teeth acidification	Paste for mouth washing and caries prevention	Abrasive cleaners for farms, hospitals and sewages
Water disinfecting filters	Ceramic membranes coated with Ag/GrO nanocomposites	Eliminates *E.coli* and *S. aureus* in water	Water disinfecting	Disinfection of water for human and animal consumption and of sewage water
Graphene coated titanium	Electrodeposition of graphene on titanium	Antibacterial activity against *S. aureus* and *E. coli*. Compatibility with peripheral blood mononuclear cells	Antimicrobial Gr-based coating of a metal	Surgical materials. Posts and plates for building hospital beds, pens and cages.

released silver ions could potentially produce toxic effects or not should also be critically evaluated, but the hemocompatibility that AgNPs have shown (Hossain et al., 2019) may suggest a safe profile for such application. On the other hand, AgNP-coated maxillofacial silicone prostheses have suggested good antimicrobial abilities and good biocompatibility measured by fibroblast viability after exposition (Meran et al., 2017; **Table 2**), enhancing the notion that polymeric materials that are coated with NPs may be safe for use.

Moreover, polymers like polyurethane and polycarbonate are widely used in hospitals for residue disposal and to build masks, physical barriers, hemodialyzers, drug delivery carriers, blood oxygenators, and arterial filters, among other biomedical devices. In this way, NP-based antimicrobial coatings for such materials may also help to control AR bacteria, not only by building devices that would be used in close contact with the medical staff and patients, but also by serving as building blocks for critical points of a hospital's facilities. Consequently, both GrO reinforced polyurethane (An et al., 2013) and polycarbonate (Mahendran et al., 2016) have been tested for potential antibacterial effects, finding good results on such ability and on their safety profiles (Mejias Carpio et al., 2012). Also, AgCl-TiO$_2$ nanocomposite was demonstrated to be an excellent matrix that released silver ions to the surroundings to inhibit biofilm formation (Naik and Kowshik, 2014). These findings could be extrapolated to

AR-endemic zones, like hospitals, farms and sewages, as these materials may be used in these facilities as building blocks for pipes, hoses and panels (**Table 2**).

Most of the research regarding the coating of biomedical devices with antimicrobial NPs has been aimed at polymers, but metallic surfaces can also be coated with such substances. An example being the electrodeposition of graphene on titanium, which facilitates hydroxyapatite aggregation and possesses interesting antibacterial abilities against *S. aureus* and *E. coli*, while being compatible with PBMCs (Janković et al., 2015) (**Table 2**). In this way, we think that this development may help to fasten prostheses with an enhanced level of security, or even to augment the sterility of scalpels, among other applications, but research about NP-coatings on other materials that are more common in hospitals, farms or sewages, like aluminum or steel, may enhance the reach of such applications in the war against AR bacteria.

On the other hand, a dentifrice containing nano-silver fluoride was shown to effectively kill *S. mutans*, prevent bacterial adhesion to teeth and to control teeth acidification significantly better than sodium fluoride-based dentifrices, thus protecting the tooth enamel in an enhanced way and suggesting a potential effectiveness to prevent caries (Teixeira et al., 2018). While the authors of the study did not evaluated the toxicity of such dentifrice, we think that the complete process of mouth washing

may be able to reduce the exposition of the users to the dentifrice's nano-compounds. Nonetheless, the absorption potential of the mucosal tissues of the mouth may enhance the infusion of such NPs into the patient's system, so that studies about this kind of application should be thoroughly conducted.

As sewage water is another endemic zone for AR-development, water disinfecting devices may be another route to cope with the ongoing problem of AR bacteria. Following such line of thought, water treatment devices that are based on ceramic membranes coated with Ag/GrO nanocomposites seem to be able to reduce from 106 colony forming units (CFUs) of either *E. coli* or *S. aureus* per mL to zero (Bao et al., 2011).

On the other hand, to our notice, AuNPs have never been used for the development of NP-based contraptions, and we think that such phenomenon may be explained by the reasoning that these NPs do not possess strong antimicrobial activities on their own, rather functioning as drug carriers and deliverers. As such, designing devices based upon AuNPs may be more complicated and prone to dysfunctions, but research is needed to discard its potential uses in such field. An interesting potential application for this kind of NPs is its conjugation with existing disinfectants, as it may potentiate their absorption and ultimately enhance their efficiency. Moreover, silver and/or graphene NP-based disinfectants could act as standalone disinfectants, as they have a proven antimicrobial activity, and as disinfectants are used on inert surfaces the requirements for safety are lower than those for drugs.

On the other hand, even when the green synthesis methods for NP production using plants (Ovais et al., 2018b) and microbial enzymes (Ovais et al., 2018a) have been considered highly cost-effective and eco-friendly, NPs that are produced by top down, and especially, bottom up techniques have an edge on size consistency, sizing accuracy, and shaping (Slepička et al., 2019). Thus, if a especial shape and/or size is needed in order for the NP-modified device to work properly, cost may be a limitation for the technology. In this sense, research on the improvement of the so called green-synthesis methods may be a key to empower NP-coated devices.

CONCLUSION

Taken together, the aforementioned data suggest that NPs may be effective allies for fighting AR-bacteria, as they are not only effective against several regular bacterial species, but they have also shown good antimicrobial abilities against AR-strains. Their properties to fight AR-bacteria even extend to the ability of graphene NPs to degrade r-plasmids and to inhibit the expression of r-genes, which may limit the spread and development of AR. Nonetheless, such substances have been shown to possess a certain degree of toxicity, which renders them as poor candidates for drug development. For this reason, NPs may be best suited for the development of extracorporeal devices with antimicrobial properties, as this kind of contraptions may utilize their potential for fighting AR-pathogens without compromising human health.

Much research is needed in order to fully evaluate the potential use of the aforementioned devices in the clinical setting, but most of the research in this area aim at the development of biomedical contraptions, without extrapolations into other endemic zones for AR-bacteria. Thus, we think that veterinary hospitals, farms and sewages must not be neglected in these initiatives, as they represent important sources of AR-bacteria, and a complete environmental control of AR-pathogens in such endemic zones may be of use in the fight against the phenomenon.

Much research is needed regarding this topic as the durability of the antimicrobial coatings, the proper NP density for their optimal function, whether or not they produce nano-contamination, or even the effectiveness and safety of other NP-based contraptions (like NP-based disinfectants) have not been studied. Also, further studies warrant a correct extrapolation of the resulting devices in all the AR-endemic zones in order to provide further control of the AR-bacteria.

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New Putative Antimicrobial Candidates: *In silico* Design of Fish-Derived Antibacterial Peptide-Motifs

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Antimicrobial resistance remains a great threat to global health. In response to the World Health Organizations' global call for action, nature has been explored for novel and safe antimicrobial candidates. To date, fish have gained recognition as potential source of safe, broad spectrum and effective antimicrobial therapeutics. The use of computational methods to design antimicrobial candidates of industrial application has however, been lagging behind. To fill the gap and contribute to the current fish-derived antimicrobial peptide repertoire, this study used Support Vector Machines algorithm to fish out fish-antimicrobial peptide-motif candidates encrypted in 127 peptides submitted at the Antimicrobial Peptide Database (APD3), steered by their physico-chemical characteristics (i.e., positive net charge, hydrophobicity, stability, molecular weight and sequence length). The best two novel antimicrobial peptide-motifs (A15_B, A15_E) with the lowest instability index (-28.25, -22.49, respectively) and highest isoelectric point (pI) index (10.48 for each) were selected for further analysis. Their 3D structures were predicted using I-TASSER and PEP-FOLD servers while ProSA, PROCHECK, and ANOLEA were used to validate them. The models predicted by I-TASSER were found to be better than those predicted by PEP-FOLD upon validation. Two I-TASSER models with the lowest c-score of -0.10 and -0.30 for A15_B and A15_E peptide-motifs, respectively, were selected for docking against known bacterial-antimicrobial target-proteins retrieved from protein databank (PDB). Carbapenam-3-carboxylate synthase (PDB ID; 4oj8) yielded the lowest docking energy (-8.80 and -7.80 Kcal/mol) against motif A15_B and A15_E, respectively, using AutoDock VINA. Further, in addition to Carbapenam-3-carboxylate synthase, these peptides (A15_B and A15_E) were found to as well bind to membrane protein (PDB ID: 1by3) and Carbapenem synthetase (PDB: 1q15) when ClusPro and HPEPDOCK tools were used. The membrane protein yielded docking energy scores (DES): -290.094, -270.751; coefficient weight (CW):

−763.6, 763.3 for A15_B and A15_E) whereas, Carbapenem synthetase (PDB: 1q15) had a DES of −236.802, −262.75 and a CW of −819.7, −829.7 for peptides A15_B and A15_E, respectively. Motif A15_B of amino acid positions 2–19 in Pleurocidin exhibited the strongest *in silico* antimicrobial potentials. This segment could be a good biological candidate of great application in pharmaceutical industries as an antimicrobial drug candidate.

Keywords: antimicrobial, fish, peptides, putative, motifs

INTRODUCTION

Infections caused by drug resistant bacteria remain one of the leading causes of death worldwide (Martín-Rodríguez et al., 2016), as the potential of conventional antibiotics to combat such microbial infections fall (Tillotson and Zinner, 2017). Over 700,000 lives are lost to antimicrobial resistance annually and the number is projected to increase (O'Neill, 2014). The rate at which these microorganisms develop resistance has outpaced the rate of production of the current class of antibiotics in spite of the immense attempts by pharmaceutical industries for new antibiotics, thereby complicating the overall efforts (Huttner et al., 2013).

Several attempts like phage therapy (Moghadam et al., 2020), anti-biofilms agents (Pletzer and Hancock, 2016; Hamayeli et al., 2019), and the use of phytochemicals (Manuel et al., 2012) have been pipelined to prevent antimicrobial resistance. Antimicrobial peptides also known as host defensive proteins (HDPs) biologics are gradually gaining ground as far as countering multiple drug resistance is concerned (Fox, 2013). A case to note is Tyrothricin; the first peptide antibiotic to be clinically used in humans (Dubos, 1939). Since its discovery over six decades ago, no record of resistance has been reported against Tyrothricin (Atiye et al., 2014). Similarly, polymixin B and Colistin are among the only standing antibiotics for the treatment of multiple drug resistant bacteria including the notorious *Acinetobacter baumanni, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* as the last line antibiotics (Falagas and Kasiakou, 2005). Their ability to withstand resistance has been attributed to their non-specific mechanism of action, multiple target sites and presence of rare D-amino acids (Ageitos and Villa, 2016). They classically conform to the first mode of action by interfering with bacterial peptidoglycan cell wall biogenesis to ease cell membrane disruption (Sujeet et al., 2018; Hao et al., 2019) and as ligands for bacterial intracellular targets (Mahlapuu et al., 2016). Most antimicrobial peptides have generally recognized as safe (GRAS) status (Hancock and Scott, 2000), with little or no toxicity (Wang S. et al., 2016). These good attributes have led to an intensified search for novel peptide antibiotics from diverse forms of life.

Fish are capable of producing antimicrobial peptides of various classes including defensins, cathelicidins, hepcidins, histone-derived peptides, and piscidins (Masso-silva and Diamond, 2014; Kumar et al., 2018). These fish derived antimicrobial peptides are active against both fish and human pathogens (Hayek et al., 2013; Huan et al., 2020; Tiralongo et al., 2020). However, their low stability coupled with insufficient information about their structures has limited their pharmaceutical applicability (Okella et al., 2018), since information on protein structure and biological (motif) interaction are key for determining the stability of any active protein (Vaidya et al., 2018). Antimicrobial activity of peptides greatly relies on amino acid composition, structure and their physicochemical properties (Kêska and Stadnik, 2017). There are numerous experimentally validated fish-derived antimicrobial peptides. However, insights into the amino acid composition, peptide structure and the target interactions with motifs in these antimicrobial peptides are lacking and present a gap that needs to be understood. This gap can however be filled through the use of *in silico* approaches. In this study we report findings of motif design, target identification and target interactions with putative antimicrobial peptide motif derived from fish.

MATERIALS AND METHODS

Study Design

This was an *in silico* study setup involving fishing out novel antimicrobial peptide motifs encrypted in 127 fish antimicrobial peptides on Antimicrobial Peptide Databases. Potential antimicrobial peptide motifs were then selected based on their physicochemical characteristics like hydrophobicity, stability, and molecular weight/size as well as sequence length. The best two antimicrobial peptide candidate-motifs were designed for their putative antimicrobial leads and docked against the known antimicrobial protein-targets to predict their potential mode of action.

Retrieval of Antimicrobial Peptide Sequence

Out of the 127 existing antimicrobial peptide (AMP) sequences, a total of 24 naturally occurring peptides (<100 amino acid residues) of fish origin (**Table 1**), with well characterized antimicrobial activity were retrieved from Antimicrobial Peptide Database (APD3) using fish as the source organism at http://aps.unmc.edu/AP/tools.php (Retrieved on May 19th, 2019) (Wang G. et al., 2016).

Antimicrobial Peptide-Motif Design

To generate and identify potential antimicrobial peptide motifs, the retrieved sequences in *FASTA* file format were subjected to web-based Support Vector Machines (SVMs) algorithm based

TABLE 1 | Retrieved fish-derived antimicrobial peptide.

APD ID	Name of peptide	Source (spp.)	Amino acid length	AMP family
AP00492	Misgurin	*Misgurnus anguillicaudatus*	21	Piscidin
AP00555	Parasin I	*Parasilurus asotus*	19	Not reported
AP00691	HFIAP-1	*Myxine glutinosa*	37	Cathelicidin
AP00692	HFIAP-3	*Myxine glutinosa*	30	Cathelicidin
AP01619	HbbetaP-1	*Ictalurus punctatus*	33	Not reported
AP01648	Pelteobagrin	*Pelteobagrus fulvidraco* R.	22	Not reported
AP01796	saBD	*Sparus aurata*	42	Defensin
AP02159	Chionodracine	*Chionodraco hamatus*	22	Piscidin-like
AP02521	PaLEAP-2	*Plecoglossus altivelis*	41	Not reported
AP02982	RP6	*Oplegnathus fasciatus*	15	Not reported
AP02983	RP7	*Oplegnathus fasciatus*	21	Not reported
AP00473	Piscidin 1	*Morone saxatilis*	22	Piscidin
AP00474	Piscidin 3	*Morone saxatilis*	22	Piscidin
AP02050	sb-Moronecidin	*Morone saxatilis*	23	Piscidin
AP00166	Pleurocidin	*Pleuronectes americanus*	25	Pleurocidin
AP02219	Cod-β defensin	*Gadus morhua*	38	Defensin
AP01713	CodCath	*Gadus morhua*	67	Cathelicidin
AP00537	SAMP H1	*Salmo salar*	30	Not reported
AP00411	Oncorhyncin II	*Oncorhynchus mykiss*	69	Not reported
AP00489	Hipposin	*Hippoglossus hippoglusus* L.	51	Not reported
AP00644	Pardaxin 4	*Pardachirus marmoratus*	33	Not reported
AP00302	Hepcidin	*Morone chrysops*	21	Hepcidin
AP02049	wb-Moronecidin	*Morone saxatilis*	*23*	Piscidin
AP02521	PaLEAP-2	*Plecoglossus altivelis*	*41*	Not reported

tool of Collection of Anti-Microbial Peptides (CAMP$_{R3}$) server (May, 2019)[1] (Waghu et al., 2016). The generated motifs were then screened based on several physiochemical parameters (Torrent et al., 2012a). The choice of the physiochemical parameters took into account that of the already existing polycationic and amphipathic AMPs; Amino acid length (18 residues), positive net charge (+4 to +6), hydrophobicity (40 and 60%) and isoelectric point of up to 10 (Wang S. et al., 2016; Hincapié et al., 2018). Helical wheels for the generated motif sequences were determined using HeliQuest server[2] at 18 amino acid window and one turn size (Gautier et al., 2008), so as to come up with cationic and hydrophobic amino acids, hydrophobicity and hydrophobic moment among other characteristics of the potential motifs (Torrent et al., 2012b). Furthermore, the instability of the putative peptides was checked using an *ExPASy* tool; *ProtParam*[3], where an instability index above zero implies it's an unstable peptide.

Antimicrobial Peptide-Motif 3D Structure Prediction and Evaluation

Due to the shortness of the peptide sequences (<30 amino acids) coupled with the absence of their experimentally attained structure for templates, the three dimensional structure of putative peptide-motifs were predicted using the Iterative Threading Assembly Refinement (I-TASSER) server[4] (Yang and Zhang, 2015). The peptides were modeled using protein templates identified by Local Meta-Threading Server (LOMETS) from the Protein Data Bank (PDB) library. LOMETS uses multiple threading approaches to align the query protein amino acid sequence against the PDB[5]. Template proteins with the highest sequence identity and lowest *Z*-score were used in the modeling exercise (**Table 2**). The best models were identified based on their c-scores. This score is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. It ranges from -5 to 2, where a lower score value indicates a highly confident model while the higher indicates the reverse. The peptide 3D structure prediction exercise was cross-validated using a web-based *de novo* peptide structure prediction tool, PEP-FOLD *v3.5*[6] (Thévenet et al., 2012). Briefly the query peptide amino acid sequences in *FASTA* format were used as the input file sequences. The algorithm was set to run 100 simulations and the output models were ranked based on sOPEP energies of individual model, where the lower the energy the better the model. The best models for both peptides A15_A and A15_B from the two peptide structure prediction tools (I-TASSER and PEP-FOLD v3.5) were then analyzed for their quality. Validation of these peptides structure was carried out in three phases;

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[1]http://www.camp.bicnirrh.res.in
[2]https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py
[3]https://web.expasy.org/protparam/

[4]https://zhanglab.ccmb.med.umich.edu/I-TASSER/
[5]http://www.rcsb.org/
[6]https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/

	A15_B					A15_E			
SN	PDB-Id	Iden1	Iden2	Cov	N Z-score	PDB-Id	Iden1	Iden2	Cov	N Z-score
1	2la2A	0.59	0.5	0.94	1.75	1rimA	0.28	0.28	1	1.66
2	6g65A	0.28	0.28	1	1.1	6mzcE	0.17	0.22	1	1.09
3	6cfz	0.45	0.28	0.61	1.02	1rimA	0.28	0.28	1	1.51
4	1tf3A	0.35	0.33	0.94	2.09	2la2	0.35	0.5	0.94	1.04
5	2kfqA	0.22	0.28	1	1.62	3bzlA	0.07	0.06	0.83	1.64
6	1rimA	0.24	0.22	0.94	1.07	2la2A	0.33	0.5	1	1.59
7	3jqhA	0.11	0.11	1	1.77	3t8sA	0.22	0.33	1	1.01
8	2jpkA	0.33	0.33	1	1.6	2pq4B	0.33	0.33	1	1.31
9	1p7aA	0.18	0.28	0.94	1.01	1be3K	0.22	0.28	1	1.56
10	1jlzA	0.39	0.39	1	1.74	2juiA	0.33	0.33	1	1.58

Iden1 is the percentage sequence identity of the templates in the threading aligned region with the query sequence. Iden2 is the percentage sequence identity of the whole template chains with query sequence. Cov represents the coverage of the threading alignment and is equal to the number of aligned residues divided by the length of query protein. N Z-score is the normalized Z-score of the threading alignments. Alignment with a Normalized Z-score >1 mean a good alignment and vice versa.

First by using Protein Structure Analysis (ProSA) web-server[7] (Wiederstein and Sippl, 2007) which predicts the query protein z-score, local model quality, and residue energy. The Z-score indicates the model quality by comparing the query protein z-score against the z-score of experimentally validated proteins available in the protein data bank (PDB). In the second phase, PROCHECK was then used to measure the stereo-chemical properties of the modeled peptide-motifs (Laskowski et al., 1993), and finally, Atomic Non-Local Environment Assessment (ANOLEA) web server[8] was used to calculate the energy of the query protein and evaluate their heavy atomic Non-Local Environment (NLE) in each molecule (Melo et al., 1997).

Target Fishing

To identify the most probable target-proteins of the motifs, all the approved antibiotic targets in the *DrugBank* database (Law et al., 2014) at https://www.drugbank.ca/targets were fished using key words; target and antibiotics. The receptor proteins alongside their identities were later retrieved from Protein Data Bank (PDB) library.

Molecular Docking Studies

The docking exercise was carried out on the top two potential AMP motifs against known protein drug targets. Docking was carried-out using the AutoDock VINA (Trott and Olson, 2019) on the DINC 2.0 Web server[9] (Antunes et al., 2017). The docking was validated using two docking tools; Hierarchical flexible Peptide Docking (HPEPDOCK) and ClusPro (Kozakov et al., 2017; Zhou et al., 2018) for optimized protein-peptide interaction. HPEPDOCK predicts the protein-peptide interaction using the hierarchical algorithm between the protein and the peptide 3D structure while ClusPro performs a global docking procedure in four folds, motif-based prediction based on peptide conformation, rigid-body docking, scoring based on

structural clustering; and final structure minimization. Briefly, the 3D structures of both the receptor protein (retrieved from PDB) and the modeled 3D peptide structures were the input files for both docking tools. Both ClusPro and HPEPDOCK docking were performed onto their respective web servers [10,11].

RESULTS

Sequence Retrieval

A total of 127 fish derived peptide sequences were retrieved out of which, 24 peptide sequences were qualified (**Table 1**). The average peptide-amino acid length was 32 residues (ranging from 15–69 residues). 20% of the retrieved peptide-sequences belonged to the cathelcidin family with 45.8% not reported. The target organisms of the retrieved peptides ranged from bacteria to yeast and fungi.

Antimicrobial Peptide Motif Design

A total of 361 peptide-motif sequences were designed from the qualified sequences which had suitable physico-chemical properties *viz.* mean hydrophobicity (H_m) greater than 0.3 (based on Fauchere and Pliska scale) (Fauchere and Pliska, 1983), net charge of + 4 and above, low instability index below zero, high antimicrobial probability were qualified. Seven peptide-motifs (**Table 3**), from which two peptide-motifs (A15_B and A15_E) with the highest stability (least instability index −28.25, −22.49, respectively) and highest antimicrobial probability (0.982) were selected for docking studies. Both peptides were found to be from the sequence of Pleurocidin; an AMP secreted by a winter flounder fish, *P. americanus* located between amino acids 2–19 and 5–22, respectively.

Peptide Motifs 3D Structure Prediction and Evaluation

The I-TASSER modeling returned five models for each modeled peptide motif (A15_B and A15_E),while the PEP-FOLD prediction returned 10 models. The best I-TASSER models had a negative c-score. I-TASSER Model-1 for both peptides (A15_B and A15_E) had the best c-score of -0.10 and -0.03, respectively (**Table 4**). On the other hand PEP-FOLD model1 for both peptides (A15_B and A15_E) were recognized as the best model with the lowest sOPEP energy of −25.1325 and −25.4534 and Apollo predicted melting temperature (tm) score of 0.703 and 0.714, respectively. The Model1_A15_B and Model1_A15_E for both I-TASSER and PEP-FOLD were characterized as the best models from both tools, thus selected for model structure analysis.

The Ramachandran plot analysis indicates that I-TASSER Model1_A15_E had 13 residues in the most favorable region and 1 in the additional allowed region. None of the Model1_A15_E peptide-motif residues were in the disallowed region. Similarly, I-TASSER Model1_A15_B had 12 residues in the most favorable region, 1 in the additional allowed region with none in disallowed region (**Figure 1**; Laskowski et al., 1993). On the other hand, PEP-FOLD model1_A15_B had 13 residues in the favorable region while PEP-FOLD model1 A15_E had 14 residues in the favorable region. In addition, a cross-validation with ProSA, showed that I-TASSER models had a z-score of -1.5, -1.27 against A15_B and A15_E, while a z-score of -1.44, -1.5 were observed for PEP-FOLD A_15_B and A_15_E models, respectively. All model z-scores were in the same range with the z-score of experimentally validated proteins, thus considered to be accurate. Likewise, ANOLEA showed that majority of I-TASSER models (33.3 and 44.5% for model A15_B and A15_E, respectively) had amino acid residues of the peptide chain in a favorable energy environment (with low energy-scores) (**Figure 2**) while PEP-FOLD model A15_B and A15_E had 22.2 and 55.7% of amino acid residues with low energy. I-TASSER model1 for both A15_B and A15_E show to be the best peptide structures and they were selected for docking exercise.

Target Fishing

A total of 28 targets were fished from the *DrugBank* database, out of which 18 had experimentally determined structures deposited at PDB (**Table 5**). Majority of the structures (83.3%) were determined using X-ray diffraction with only one structure (C-1027) determined using solution Nuclear Magnetic Resonance (NMR).

Molecular Docking

Docking exercise with AutoDock VINA revealed that both peptide-motifs (A15_B and A15_E) were able to bind with low docking energies (ranging from -8.80 to -5.80 Kcal/mol) indicating their fairly high affinity with the selected antimicrobial target protein (**Table 6**). The best docking energy, however, was observed against *vancosaminyl transferase* protein (PDB ID; 1rrv, docking energy (DE); −8.20, −7.60 Kcal/mol), *Beta-hexosaminidase* protein (PDB ID; 4g46 DE; −7.90, −7.70 Kcal/mol), membrane protein (PDB: 1by3 DE; −7.3, −7.3), and *carbapenam* protein (PDB ID; 4oj8 DE; −8.80, −7.80 Kcal/mol) against peptide-motif A15_B and A15_E, respectively (**Table 6**). The affinity of peptide-motifs A15_B and A15_E was highest within chains of the target proteins (PDB ID 1rrv, 4g6c, and 4oj8). Docking validation with HPEPDOCK shows that membrane protein (PDB: 1by3) and Carbapenem synthetase had the highest docking potential to peptide A15_B and A15_E with a docking energy score of −290.094, −270.751 against protein 1by3 and −236.802, −262.75 against 1q15, respectively. Likewise, docking with ClusPro further indicated that membrane protein and carbapenem synthetase had the highest chance to bind to peptide A15_B and A15_E with a coefficient weight of −763.6, −763.3 against protein 1by3 and −819.7, −829.7 against peptide A15_B and A15_E, respectively. Carbapenam synthetase (PDB ID; 4oj8) which had the lowest docking energy against the two peptides was found to be among the targets with lowest docking energies scores of (-221.657 and −196.952) against peptide A15_B and A15_E using HPEPDOCK. However, this protein had the lowest coefficient weight score of −681.2 and −66.8 against peptide A15_B and A15_E using ClusPro, respectively. Peptide motif A15_B which had the lowest instability index (highest stability) also showed a relatively higher binding affinity than its counterpart A15_E (**Table 6**) in all the 3 docking methods, except with protein 1by3 where peptide A15_E had a lower docking energy score than A15_B using HPEPDOCK.

DISCUSSION

The present study demonstrates that an online Support Vector Machines (SVMs) algorithm effectively localizes motifs of potentially best antimicrobial activity within a

TABLE 3 | Physicochemical properties of peptides sourced through *in silico* analysis.

Peptide	Sequence	Charge	H (%)	H_m	$\mu\, H_r$	pI	Ii	MW (Da)	A_P
A15_B	WGSFFKKAAHVGKHVGKA	+4	44.44	0.303	0.508	10.48	−28.25	1955.30	0.982
A15_E	FFKKAAHVGKHVGKAALT	+4	50.00	0.307	0.347	10.48	−22.49	1910.30	0.982
A20_Q	RSSRAGLQFPVGRVHRLL	+4	44.44	0.342	0.349	12.48	85.86	2049.41	0.741
A20_R	SSRAGLQFPVGRVHRLLR	+4	44.44	0.342	0.349	12.48	72.15	2049.41	0.729
A20_U	AGLQFPVGRVHRLLRKGN	+ 4	44.44	0.314	0.380	12.30	41.32	2018.40	0.917
A20_V	GLQFPVGRVHRLLRKGNY	+4	44.44	0.350	0.344	11.72	41.32	2110.50	0.816
A20_W	LQFPVGRVHRLLRKGNYA	+4	50.00	0.367	0.346	11.72	54.47	2124.52	0.740

H, Hydrophobicity; Hm, mean hydrophobicity; μHr, relative hydrophobic moment; Ii, instability index; MW, molecular weight; A_P, Antimicrobial probability.

TABLE 4 | Top 5 output peptide structure prediction models from i-TASSER, PEP-FOLD, and their model evaluation.

Models	iTASSER output modelC-score		PEP-FOLD output model scores			
	A15_B	A15_E	A15_E		A15_B	
			sOPEP	tm	sOPEP	tm
Model1	−0.10	−0.03	−25.4534	0.703	−25.1325	0.714
Model2	−5	−5	−25.3043	0.661	−25.0347	0.740
Model3	−5	−5	−25.2665	0.716	−25.0096	0.764
Model4	−5	−5	−25.0894	0.694	−24.8657	0.760
Model5	−5	−1.76	−24.895	0.670	−24.7082	0.739

FIGURE 1 | I-TASSER predicted peptide 3D structure homology models and their Ramachandran validation plots. **(A)** A15_B peptide-motif, **(B)** Ramachandran plot for A15_B peptide-motif, **(C)** A15_E peptide-motif, **(D)** Ramachandran plot for A15_E peptide-motif. Peptide-motif A15_B had 12 amino acids sequences in the allowed region while peptide-motif A15_E had 13 amino acid in the favorable region. Both peptide-motifs had no amino acid sequence in the disallowed region. The cartons were rendered in *Edu PyMOL*.

FIGURE 2 | I-TASSER predicted peptide 3D structure ANOLEA and ProSA validation plots. **(A)** Peptide A15_B ANOLEA energy score, **(B)** Peptide A15_B ProSA z-score, **(C)** Peptide A15_E ANOLEA energy score, **(D)** Peptide A15_B ProSA z-score. ANOLEA validation showed that 33.3 and 44.5% of peptide A15_B and A15_E had their amino acid residues in the favorable regions (low energy scores highlighted in red). Peptide motifs A15_B and A15_E had z-scores of −1.5 and −1.27, respectively, and were within the normal z-score of experimentally validated proteins. The ANOLEA plots were generated in R using latticeExtra package.

TABLE 5 | Antimicrobial target proteins used in the docking exercise.

Protein name	PDB Id	Classification	Organism	Method
C-1027	1hzl	Antibiotic	*Streptomyces globisporus*	Solution NMR
Tyrosine aminomutase	3kdy	Lyase	*Streptomyces globisporus*	X-ray diffraction
50s ribosomal protein l32	6qul	Antibiotic	*Escherichia coli*	Electron microscopy
Carbapenam synthetase	1q15	Biosynthetic protein	*Pectobacterium carotovorum*	X-ray diffraction
Iron(3 +)-hydroxamate-binding protein fhud	1esz	Metal transport	*Escherichia coli*	X-ray diffraction
Fhua	1by3	Membrane protein	*Escherichia coli*	X-ray diffraction
Neocarzinostatin	1nco	Antibacterial and antitumor protein	*Streptomyces carzinostaticus*	X-ray diffraction
Protein phzg	1ty9	Oxidoreductase	*Pseudomonas fluorescens*	X-ray diffraction
Lipocalins	1nyc	Hydrolase inhibitor	*Escherichia coli*	X-ray diffraction
D-alanyl-d-alanine carboxypeptidase	6osu	Hydrolase	*Francisella tularensis* subsp. *Tularensis schu* s4	X-ray diffraction
Beta-hexosaminidase	4g6c	Hydrolase	*Burkholderia cenocepacia* j2315	X-ray diffraction
Mexa of the multidrug transporter	1vf7	Membrane protein	*Pseudomonas aeruginosa*	X-ray diffraction
S/t protein kinase pkng	4y0x	Transferase	*Mycobacterium tuberculosis* h37rv	X-ray diffraction
Bacterial 45srbga ribosomal particle class a	6pvk	Ribosome	*Bacillus subtilis*	Electron microscopy
Neocarzinostatin	1nco	Antibacterial and antitumor protein	*Streptomyces carzinostaticus*	X-ray diffraction
Carbapenam	4oj8	Oxidoreductase	*Pectobacterium carotovorum* subsp. Carotovorum	X-ray diffraction
Vancosaminyl transferase	1rrv	Transferase/antibiotic	*Amycolatopsis orientalis*	X-ray diffraction

TABLE 6 | Docking energies and score of ligand A15_B, A15_E against the Antimicrobial target proteins using Autodock Vina, HPEPDOCK, ClusPro.

DB ID	Center AA	Energies with AutoDock VINA (Kcal/mol)		Energy scores with HPEPDOCK		Coefficient weight score with ClusPro	
		A15_E	A15_B	A15_E	A15_B	A15_E	A15_B
1by3	HIS-89	−7.30*	−7.30*	−290.094*	−270.751*	−763.6*	−763.3*
1e5z	PHE-274	−5.80	−6.40	−201.893	−202.313	−652.9	−766.9
1hzl	GLN-35	−5.40	−5.40	−192.021	−181.348	−594.3	−617.6
1kny	GLN-168	−7.10	−7.20	−186.724	−198.732	−767.7	−802.2
1nco	ALA-2	−6.20	−6.70	−199.495	−183.348	−678.9	−753.1
1nyc	TRP-31	−6.70	−6.60	−216.461	−206.614	−651.8	−791.5
1q15	ARG-50	−7.00	−6.80	−236.802*	−262.750*	−819.7*	−829.7*
1rrv	ALA-265	−7.60*	−8.20*	−208.564	−179.493	−761.8	−769.3
1ty9	VAL-108	−6.90	−6.60	−221.560	−196.827	−652.9	−677
3kdy	ASP-366	−6.10	−6.10	−233.213	−208.320	−663.6	−721.9
4g6c	HIS-158	−7.70*	−7.90*	−182.505	−213.155	−602.0	−700.4
4oj8	ALA-144	−7.80*	−8.80*	−221.657*	−196.952*	−681.2	−666.8
6osu	VAL-32	−6.20	−6.10	−182.232	−198.953	−511.7	−610.1

AA, Amino acid. *Proteins with the lowest docking energies. Lowest energies against protein with the highest probability to dock to peptide A15_B and A15_E.

peptide. This technique is vital in enhancing the antimicrobial activity of peptides especially on resistant strains including *Pseudomonas aeruginosa* (Torrent et al., 2012c). The strength of this study is hinged on its ability to generate very many peptide fragments and being able to systematically sieve them based on their physicochemical parameters to arrive at the best candidates. However, the number of peptide templates used was small 24 (0.77%) compared to a total of 3,105 antimicrobial peptides in the antimicrobial peptides database (accessed on 01.08.2019). This is due to the fact that this study focuses only on "experimentally validated" peptides even so, only 127 fish antimicrobial peptides are present at the database.

Out of the 361 peptide motifs generated, the most active with the highest *in silico* antimicrobial probability of 0.982 (A15_B and A15_E) were both from Pleurocidin; an AMP secreted by flatfish, *Pleuronectes americanus* that largely inhabits soft muddy to moderately hard bottoms of marine waters. Even so, motif A15_B proved to be much more stable (instability index −28.25), rendering it the best fragment designed. When docked with AutoDock VINA, A15_B continued as the best designed peptide motif yielding the highest binding energy (−8.80 Kcal/mol) and highest number of hydrogen bond interactions (3) on Carbapenam-3-carboxylate synthase target. This indicates the motif (A15_B) binds spontaneously onto Carbapenam-3-carboxylate synthase target without consuming energy (Meng et al., 2011). Moreover, docking with HPEPDOCK and ClusPro further indicated that Carbapenam synthetase protein (PDB: 1Q15) alongside a Membrane proteins (PDB: 1by3) and Carbapenam-3-caboxylate protein (PDB: 4oj8) are among the proteins with highest binding potentials to peptide motif A15_B. However, Carbapenam-3-caboxylate protein yielded the least Docking energy when compared to the Membrane proteins and carbapenam synthetase and Carbapenam synthetase protein.

Carbapenam-3-carboxylate synthase is responsible for the biosynthesis of the naturally occurring β-lactam antibiotics in bacteria (Stapon et al., 2003). The enzyme catalyzes the ATP-dependent formation of (3S,5S)-carbapenam-3-carboxylate from (2S,5S)-5-carboxymethylproline in *Pectobacterium carotovorum* (Gerratana et al., 2003). Therefore, the binding of the designed peptide motif A15_B is likely to activate Carbapenam-3-carboxylate synthase to synthesis amass of natural antibiotic that destroys the bacteria (Samantha et al., 2007), a phenomenon that can be explored for novel therapeutics. However, being a novel motif on amino acids of positions 2–19 of Pleurocidin, this study could hardly access preceding studies to match the complex binding affinity.

An important but unanswered question is how these peptides can be optimized for a good platform particularly in drug discovery where the nature and properties of potential hits can be understood specifically on how best they can be modified into useful leads as antimicrobials in the fight against drug resistance. Ultimately, efforts are underway for better ways to handle such small fragments on benches to ascertain the *in vitro* and *in vivo* efficacy in low resource facilities.

CONCLUSION

This study revealed that the motifs (A15_B) of amino acid positions 2-19 in Pleurocidin secreted by a winter flounder fish, *Pleuronectes americanus* as the best antimicrobial potentials. This segment is among the promising biological candidates that could be of great application in pharmaceutical and nutraceutical industries as virtual tools show great potentials in drug development even in the absence of large investment laboratory equipment. However, further studies focused on synthesized peptides would be helpful.

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/s.

AUTHOR CONTRIBUTIONS

HO, SO, and CN designed and implemented the study. JA, CA, HI, FF, JN, CK, CB, PO, HO, AM, JG,

and KK performed the experiments and data analysis. All authors participated in writing and proofreading the manuscript and approved the final manuscript for publication.

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