Examining the Antibiotic Potentiating Activity of Cranberry-Derived Proanthocyanidins Against β-Lactam Resistant Bacteria

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Contribution of Authors

This thesis contains four main chapters: chapter 1 introduces the background of the thesis; chapter 2 summarizes the literature review and the important related research works; chapter 3 consists of the journal article manuscript entitled “Cranberry-Derived Proanthocyanidins Potentiate β-Lactam Antibiotics Against Resistant Bacteria”; chapter 4 contains the conclusion of the thesis and recommendations for future work.

Kuan Wei composed chapter 1, chapter 2, and chapter 4. The manuscript in chapter 3 is co-authored by Mathias Gallique, Kuan Wei, Vimal Maisuria, Mira Okshevsky, Geoffrey McKay, Dao Nguyen, and Nathalie Tufenkji. Mathias Gallique, Kuan Wei, Vimal Maisuria, Mira Okshevsky, Dao Nguyen and Nathalie Tufenkji conceived the experiments. Mathias Gallique and Kuan Wei contributed equally to the design of experiments, most of the experimental work, data analysis, and drafting of the manuscript. Specifically, Mathias Gallique and Kuan Wei collaboratively constructed *mecA*-expressing Methicillin-susceptible *Staphylococcus aureus* strain and *blaZ*-expressing *Escherichia coli* strain. Mathias Gallique performed the majority of experiments and data analysis regarding quantitative RT-PCR and checkerboard microdilution assays. Kuan Wei primarily focused on experiments and data analysis on β-Lactamase activity assays. Vimal Maisuria performed the preliminary experiments and analysis for the project. Geoffrey McKay provided valuable advice and supervision. Dao Nguyen and Nathalie Tufenkji offered essential expertise and guidance throughout the project. All co-authors participated in the revision of the manuscript.

Abstract

Before the 1930s, bacterial infections were the leading cause of human death. Diseases due to bacterial infections, such as cellulitis, pneumonia, and endocarditis, had very high mortality rates. Thankfully, in the 1930s, antibiotics – medicines that can effectively treat bacterial infections - were introduced to the public. Until now, antibiotics are still one of the most powerful tools against bacterial infections. However, the spread of antibiotic resistance is seriously weakening their effectiveness, and causing enormous social and economic burdens.

Among the most commonly used antibiotics are β-lactam antibiotics, with an annual expenditure equivalent to ~65% of the total antibiotic market. Antibiotic resistance against β-lactam antibiotics is therefore particularly detrimental to public health. β-lactam antibiotics inhibit the growth of bacteria by irreversibly occupying the active sites of penicillin-binding proteins (PBPs), the proteins that are crucial in the cell wall synthesis of bacteria. Two major mechanisms of β-lactam resistance exist: the production of β-lactamases and the synthesis of PBP2a. β-lactamases are enzymes that can disable the functions of β-lactam antibiotics through hydrolyzing their functional structures. PBP2a is a type of structurally altered PBP that has a very low affinity with β-lactam antibiotics. Thousands fold more β-lactam antibiotics are required to kill bacteria harboring these two resistance mechanisms. Solutions to combat β-lactam resistant bacteria and mitigate the spread of β-lactam resistance are thus urgently needed.

This thesis provides evidence for a natural polyphenol - cranberry-derived proanthocyanidins (cPAC) – as an antibiotic potentiator against β-lactam resistant bacteria. We demonstrated that cPAC could significantly potentiate β-lactam antibiotics against β-lactam resistant *Enterobacteriaceae* clinical isolates as well as Extended-Spectrum-β-lactamase- and metallo-β-lactamase-producing *Escherichia coli*. Using nitrocefin assays, an *in vitro* assay to measure β-lactamase activity, we further identified the potentiation was, in some cases, due to the inhibition of the β-lactamase activity by cPAC. We also found that cPAC can potentiate oxacillin and carbenicillin, leading to significant decreases in the concentrations of the antibiotics required to inhibit the growth of methicillin-resistant *Staphylococcus* spp.. By genetically transforming the *mecA* gene into a methicillin-susceptible *Staphylococcus aureus* strain, we further showed that cPAC interfered with the PBP2a-associated resistance pathway. These discoveries demonstrate the

potential of cPAC in overcoming β-lactam antibiotic resistance, and provide the motivation for further investigation in cPAC's medical value.

Résumé

Avant les années 30, les infections bactériennes étaient la principale cause de mortalité chez l'être humain. En effet, les maladies associées aux infections bactériennes, telle que la cellulite infectieuse, la pneumonie ou l'endocardite, présentaient un fort taux de mortalité. Les antibiotiques, médicaments permettant de soigner ces types d'infections, ont été présenté au public. Jusqu'à maintenant, les antibiotiques sont considérés comme l'un des outils les plus efficaces pour lutter contre les infections bactériennes. Cependant, l'émergence de la résistance aux antibiotiques, menaçant sérieusement leur efficacité, représente un lourd fardeau économique et social.

Avec une dépense annuelle estimée à environ 65% de la part totale du marché des antibiotiques, les β-lactamines font partie des antibiotiques les plus fréquemment utilisés. Les β-lactamines inhibent la croissance bactérienne en se fixant de manière irréversible sur les protéines de liaison aux pénicillines (PLPs), protéines essentielles dans la synthèse de la paroi cellulaire bactérienne. La résistance à ces antibiotiques est une sérieuse menace pour la santé publique. Deux mécanismes majeurs sont associés à la résistance aux β-lactamines : la production de β-lactamases et l'acquisition de la transpeptidase altérée PBP2a. Les β-lactamases sont des enzymes qui dégradent la structure fonctionnelle des β-lactamines, inactivant ainsi leurs fonctions antimicrobiennes. PBP2a est une PLP altérée qui possède une faible affinité pour les β-lactamines. L'acquisition d'un de ces mécanismes de résistance nécessite des quantités plus importantes d'antibiotiques pour tuer ces bactéries de manière efficace. Il est donc urgent de trouver des solutions pour faire face à l'émergence des résistances aux antibiotiques et de limiter ainsi leur propagation.

Cette thèse met en évidence que les polyphénols naturels - les proanthocyanidines dérivées de la canneberge (cPAC) - peuvent être utilisées comme potentiateur d'antibiotiques contre des bactéries résistantes aux β-lactamines. Nous avons démontré que les cPAC peuvent significativement potentialiser certaines β-lactamines contre des Entérobactéries résistantes à ces dernières, mais également des souches d'*Escherichia coli* produisant des β-lactamases à spectre étendu ou bien des metallo-β-lactamases. En mesurant l'activité des β-lactamases *in vitro* avec les tests de nitrocéfine, nous avons mis en évidence que dans certains cas la potentialisation était

associée à l'activité inhibitrice des cPAC sur les β-lactamases. Nous avons également montré que les cPAC peuvent fortement diminuer la concentration en oxacilline et carbénicilline requise pour inhiber la croissance de *Staphylococcus aureus* résistant à la méticilline. En introduisant le gène *mecA* codant pour PBP2a chez une souche de *S. aureus* sensible à la méticilline, nous avons pu montrer que les cPAC interfèrent avec le mécanisme de résistance conféré par PBP2a. Ces découvertes démontrent le potentiel des cPAC pour vaincre la résistance aux β-lactamines, ce qui motive encore davantage la valorisation des cPAC dans le milieu médical.

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1 Introduction

In the 1930s, the first antibiotics became commercially available to the world. Since then, antibiotics have helped humankind fight against numerous diseases caused by a broad range of bacterial infections[1]. In 1942, the introduction of penicillin to the public marked the beginning of the "golden era" of antibiotic discovery, when the majority of antibiotics that we are still using nowadays were discovered and synthesized[1]. However, the flourishing age did not last long. After 1962, the process of developing new antibiotics slowed down. Since the latest class of antibiotics (lipopeptides) was discovered and introduced to the market in 1987, there has been no novel class of antibiotics reaching the market[2].

Paradoxically, one of the primary reasons that hinder the discovery of new antibiotics is the growing number of resistant bacteria. The overuse of antibiotics leads to selective pressure on bacteria and accelerate the development of resistance[3]. To control the spread of antibiotic resistance, new antibiotics are often prescribed for only the most severe illnesses. While the restrain in the use of new antibiotics can slow down the evolution of resistance, it also diminishes the return on investment. Besides, as the emergence of resistance is almost inevitable, the profit of developing a new antibiotic becomes uncertain[3]. As a result, fewer pharmaceutical professionals consider the investment in antibiotics as an economically wise choice. The limited sources of funding eventually cause the stagnation in the development of novel antibiotics[4].

One class of antibiotics that has greatly suffered from antibiotic resistance is β-lactams. Originated from penicillin, β-lactams have been the most widely used class of antibiotics for decades, due to their high effectiveness against a broad range of Gram-negative and Gram-positive bacteria and minor side-effects compared to other antibiotics[5]. However, their extensive usage also causes the development of resistance in a wide range of common pathogenic bacteria such as *Escherichia coli* (*E. coli*)[6–9], *Staphylococcus aureus* (*S. aureus*)[10–12], and *Klebsiella pneumoniae* (*K. pneumoniae*)[8,13,14]. The resistance can dramatically increase the antibiotic usages for treating infections caused by these pathogens and thus cause elevated risks of side-effects and higher economic burdens to patients. Solutions that can effectively treat resistant pathogens and reduce the spread of β-lactam antibiotic resistance are thus urgently required.

Antibiotic potentiators, also known as antibiotic adjuvants, are arguably the most common and successful weapons to overcome antibiotic resistance to date[15]. These small molecules are often not antibacterial by themselves but can inhibit the resistance mechanisms of bacteria. Through co-administration with antibiotics, antibiotic potentiators can facilitate the antibiotic activities, leading to lower required doses to

inhibit the growth of target bacteria[16]. Antibiotic potentiators are also commonly used to overcome β-lactam antibiotic resistance. For instance, the production of β-lactamases, a family of enzymes that hydrolyze β-lactam antibiotics, is a primary β-lactam antibiotic resistance mechanism[17]. A group of β-lactam antibiotic potentiators named β-lactamase inhibitors can effectively block the activity of β-lactamases. Over the last decades, they have achieved great successes in clinical uses[18–20].

One large group of antibiotic potentiators comes from the secondary metabolites of plants[21]. For a long time, humankind has been using natural products from plants as traditional folk medicine, but it is not until the mid-20th century that scientists were able to fractionate the plant extracts at the molecular level and investigate the molecules that give the plants such potencies[22]. Many plants exhibiting health benefits have been shown to contain phenolic compounds of interest (Table 1). Recent studies have also discovered that several phenolic compounds, such as epigallocatechin gallate[23] and tannic acid[24], can effectively reduce the resistance of pathogenic bacteria towards β-lactam antibiotics, and significantly decrease the β-lactams concentrations required to kill the bacteria. Hence, discovering the health benefits of natural phenolic compounds is a research area of growing interest, and this thesis aims to study the phenolic compounds extracted from the American cranberry, as the potentiator of existing β-lactam antibiotics, against β-lactam resistant pathogens.

In this first chapter, I give an overview of β-lactam antibiotics and their mechanisms. I also review the important β-lactam resistance mechanisms and relevant pathogenic bacteria. In the end, a more in-depth introduction about β-lactam antibiotic potentiators and the antimicrobial properties of cPAC is also given.

Table 1. Examples of Medical Plants Containing Polyphenolic Compounds[22]

Plant Name	Function	Principle Polyphenolic Metabolites
Tree Peony (*Paeonia lactiflora*)	outer skin of the root; used to cure disorders of the bloodstream, including high blood pressure.	gallotannins
Bearberry (*Arctostaphylos uva-ursi*)	dried leaves; infusions have a soothing astringent effect and have value as a diuretic in kidney disorders and ailments of the bladder and urinary tract.	gallotannins, arbutin, galloyl esters of arbutin
Meadowsweet (*Filipendula ulmaria*)	aerial parts of the plant, leaves and flowers used as an infusion; employed as a mild astringent, antirheumatic antiinflammatory agent, and as a diuretic.	ellagitannins

Raspberry (*Rubus idaeus*)	leaves and fruit; mild astringent used in disorders of the digestive system, raspberry leaf tea traditionally used during pregnancy.	ellagitannins
Hawthorn (*Crataegus sp.*)	leaves and berries; used as astringent for digestive system, diuretic, cardiac tonic in treatment of high blood pressure.	proanthocyanidins

1.1 Antibiotic and Antibiotic Resistance

Antibiotics are one of the most powerful weapons against bacterial infections. The use of antibiotics has led to significant decreases in mortality rates of many diseases, such as cellulitis, pneumonia, and endocarditis[25]. They are expected to add 5-10 years to U.S. life expectancy at birth, and save 200,000 American lives every year[26]. However, these miracle drugs are threatened by antibiotic resistance. Besides hindering the development of novel antibiotics, antibiotic resistance is causing serious social and economic burdens. As reported by Centers for Disease Control and Prevention (CDC), every year in the U.S., more than 2 million people are infected by antibiotic-resistant bacteria, and 23,000 people die because of the infections[27]. In addition, antibiotic resistance is estimated to cause additional medical use that costs $2.2 billion each year[28].

Antibiotic resistance can be categorized into two main types: intrinsic resistance and acquired resistance. Intrinsic resistance is a trait that can be universally found in a bacteria species[29]. Some examples are the outer membranes that have limited permeability to antibiotics in many Gram-Negative bacteria[29], and efflux pumps that are ready to eliminate the penetrated antibiotics out of the cell in *Pseudomonas aeruginosa (P. aeruginosa*)[30]. On the other hand, acquired resistance is not originally present in a bacteria species. It is acquired under selective pressure through horizontal gene transfer[31] or mutation[32]. Unlike intrinsic resistance, acquired resistance is often more specific toward one kind of antibiotics. Major resistance mechanisms against specifically β-lactam antibiotics are acquired resistance mechanisms.

1.2 β-Lactam Antibiotics and Their Mechanisms of Action

Despite a large variety of antibiotics, the primary targets of antibiotics remain within the five essential components of bacteria: cell wall, DNA/RNA synthesis, plasma membrane, ribosomes, and metabolic pathways[33]. β-lactam antibiotics are the most common antibiotics that target the bacterial cell wall.

The bacterial cell wall mainly consists of peptidoglycan, and is the vital component of bacteria that maintains their overall shape and allows them to survive under osmosis pressure[34]. Peptidoglycan is

formed through cross-linking of alternating glycan strands, N-acetylmuramic Acid (NAM) and N-acetylglucosamine (NAG), via short peptide bridges[35]. NAM contains a peptide side chain generally with an amino acid sequence of L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. The cross-linking process is catalyzed by transpeptidases, which facilitates the formation of short peptide bridges between the carboxyl group of D-Ala at position 4 and the amino group of the diamino acid at position 3[36]. The cross-linking is essential for the rigid structure of the cell wall. Besides being a protective layer, the cell wall also has to be flexible enough for cellular expansion and division[37]. This is achieved through a group of enzymes named "autolysin", which are responsible for cleaving or modifying the peptidoglycan structure[38]. The remodeling and the formation of peptidoglycan need to be carefully balanced to ensure the growth and proliferation of bacteria[39].

All β-lactam antibiotics share a common core structure of a four-member-β-lactam ring (Figure 1)[40]. As stated above, D-Ala-D-Ala is a substrate for transpeptidases. Therefore, transpeptidases have a high affinity to D-Ala-D-Ala. Because penicillin, and β-lactam antibiotics in general, are structurally similar to D-Ala-D-Ala, they can also bind to the transpeptidases. The transpeptidases with a high affinity towards the β-lactam antibiotics are referred to as penicillin-binding protein (PBP). After binding to the PBPs, the β-lactam antibiotics irreversibly acylate the serine on the active sites of these transpeptidases, and form a stable acyl-enzyme complex, as opposed to the transient acyl-enzyme complex derived by D-Ala-D-Ala. This process results in the lack of free active sites on PBPs for D-Ala-D-Ala, and thus impairs peptidoglycan formation[10,41]. Subsequently, the balance between the remodeling and the formation of peptidoglycan is broken, causing the hydrolysis process to outpace the formation process. The decrease in the amount of peptidoglycan leads to a compromised cell wall, which loses the ability to protect the integrity of the cell, and eventually causes cell lysis[42].

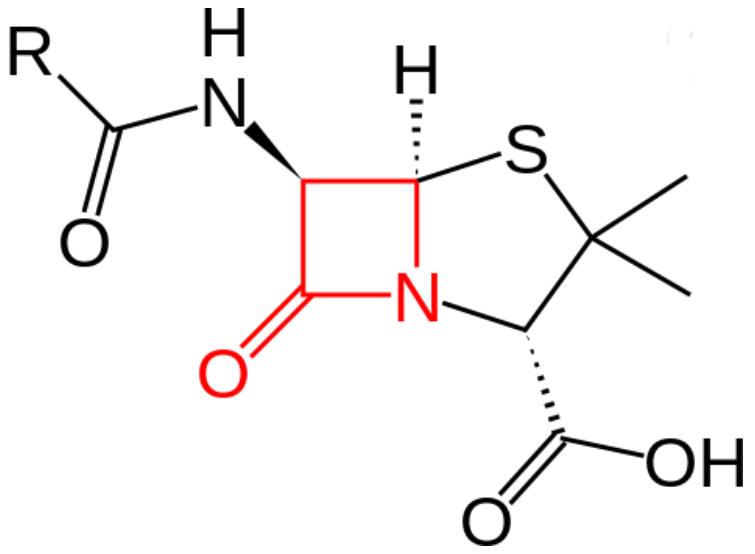

Figure 1. A penicillin core structure. The four-member-β-lactam ring is highlighted in red[43].

1.3 β-Lactam-Resistance Mechanisms

Due to their high effectiveness and relatively safe profile, β-lactam antibiotics are some of the most commonly prescribed drugs in clinics[44], with their annual expenditure contribute to 65% of the total antibiotic market[45]. The overuse of β-lactam antibiotics inevitably leads to the development of resistance mechanisms.

Two primary mechanisms of β-lactam resistance are currently known. The first mechanism is the production of β-lactamases, a type of enzyme that can hydrolyze the β-lactam ring. As described above, PBPs only recognize the four-member-β-lactam ring in the β-lactam antibiotics. Through the hydrolysis of the four-member-β-lactam ring, β-lactamases prevent the β-lactam antibiotics from binding to the PBPs, and subsequently render the antibiotics dysfunctional[40]. The production of β-lactamases is the most common β-lactam resistance mechanism, especially in Gram-negative pathogens, such as Enterobacteriaceae[46]. β-lactamases can be structurally separated into four classes[47]: Class A, Class B, Class C, and Class D. Class A, Class C, and Class D β-lactamases are distantly related to PBPs as they share an invariant Ser–Xaa–Xaa–Lys amino-sequence pattern[46]. They use this serine as the active site to hydrolyze β-lactams. Therefore, they are also called serine-β-lactamases (SBLs)[48]. Class B β-lactamases require zinc ions on their active site to function, instead of serine[49]. Thus, Class B β-lactamases are also named metallo-β-lactamases (MBLs).

The second important mechanism is the production of structurally altered PBPs. These variant PBPs have a changed structure in their active site, which leads to much lower acylation rate by β-lactam antibiotics

and prevents the irreversible covalent binding to form[50]. At the same time, they can still function as the normal PBPs to facilitate peptidoglycan formation. Therefore, even though the normal PBPs can be inhibited in the presence of β-lactam antibiotics, these variant PBPs can continue the cell wall synthesis and maintain the bacterial proliferation. This resistance mechanism is usually found in Gram-positive bacteria, and often associates with broad-range β-lactam resistance[51]. One of the most well-known PBP variants is the PBP2a in methicillin-resistant *S. aureus* (MRSA).

1.4 Enterobacteriaceae species

Enterobacteriaceae are a large group of Gram-negative bacteria that contribute to a significant portion of nosocomial infections, including urinary tract infections (URI), respiratory infections, tissue infections, and more[52,53]. In fact, some notorious examples such as *E. coli* and *K. pneumoniae* are among the most common human pathogens[54]. Most Enterobacteriaceae are susceptible to β-lactam antibiotics, making the latter a good choice for Enterobacteriaceae-related infections[44]. However, extended-spectrum-β-lactamases (ESBL)-producing and Carbapenem-resistant Enterobacteriaceae are becoming prevalent, causing major public health threats in regions and countries[53–55]. The antibiotic resistance of Enterobacteriaceae seriously limits the options for treatments, and increases the mortality rate[56,57]. In the U.S., it was estimated that 197,400 cases of hospitalization and 9,100 deaths were caused by ESBL-producing Enterobacteriaceae. Thus, strategies to overcome the resistance in Enterobacteriaceae species are necessary.

1.5 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

The Gram-positive bacterium *S. aureus* is one of the most common causes of hospital- and community-associated infections[58–60]. Moreover, it is one of the major causes of healthcare-associated pneumonia and bloodstream infections[61,62]. Historically, *S. aureu*s was widely susceptible to β-lactam antibiotics. Along with the good safety profile, β-lactam antibiotics have been considered as the agents of choice for *S. aureus* infections. However, recent studies show an increasing number of *S. aureus* that are not susceptible to β-lactam antibiotics in both hospital- and community-associated infections[63,64]. In fact, it has been reported that around 90% of *S. aureus* colonized on humans contain penicillin resistance[65]. The development of β-lactam antibiotic resistance poses a great challenge towards preventing and treating *S. aureus* infections.

The first type of resistant *S. aureus* is called methicillin susceptible *S. aureus* (MSSA). Their resistance comes from the production of PC-1, a Class-A SBL that can effectively hydrolyze penicillin[66]. MSSA caused serious concerns in the late 1950s. To combat the resistance, methicillin – a penicillin derivative

resistant to PC-1 hydrolysis – was synthesized[67]. Methicillin was effective against the *S. aureus* that relied on the production of PC-1 as its β-lactam-resistance mechanism, and hence the name MSSA.

However, as soon as methicillin was introduced for clinical use, *S. aureus* resistant to methicillin, namely MRSA, was found. The β-lactam antibiotic resistance of MRSA comes from both the production of PC-1 and the synthesis of PBP2a[11]. MRSA possesses resistance to methicillin due to the presence of the *mecA* gene in the chromosome. The *mecA* gene is believed to be acquired by MRSA from a foreign species, and it is responsible for the synthesis of PBP2a[67]. PBP2a is a type of structurally altered PBP. As compared to endogenous PBPs, PBP2a has a peptide loop at the entrance of the transpeptidase active site, which blocks the introduction of large molecules such as β-lactam antibiotics, and makes PBP2a intrinsically resistant to β-lactam antibiotics[41]. Thus, when exposed to β-lactam antibiotics, PBP2a can substitute PBPs for the cell wall synthesis[10]. Compared to PC-1, PBP2a provides *S. aureus* a much broader spectrum of β-lactam antibiotics resistance[68].

A study[69] has shown that 44.6% of *S. aureus* isolates were methicillin resistant in 2014 in China. The resistance of MRSA could significantly complicate the treatments, and lead to an increase health care cost of above $9600 and an extension in hospital stay of 14 days per patient[69]. Due to MRSA's high clinical importance, solutions to overcome its β-lactam resistance are of urgent need.

1.6 Antibiotic Potentiation As A Solution Against β-Lactam Resistance

Antibiotic potentiators are widely used in combination with β-lactam antibiotics against β-lactamase producing bacteria. These antibiotic potentiators are known as β-lactamase inhibitors. Like other antibiotic potentiators, β-lactamase inhibitors are often not bactericidal on their own. However, when combined with β-lactam antibiotics, they can significantly lower the concentrations of antibiotics needed to inhibit resistant bacteria. Most common β-lactamase inhibitors are structurally analogous to β-lactam antibiotics, and therefore have a high affinity to β-lactamases. Unlike β-lactam antibiotics that will be hydrolyzed after binding to β-lactamases, β-lactamase inhibitors irreversibly occupy the active site of β-lactamases and hinder their function. Nowadays, many β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, have been clinically approved and demonstrated to be successful strategies to treat β-lactamase associated resistant bacteria[70].

However, β-lactamase inhibitors also have their limitations. So far, most β-lactamase inhibitors, including the ones mentioned above, are only effective against Class A enzymes, and are less effective against Class

B, Class C, and Class D enzymes[70–72]. Despite a new generation β-lactamase inhibitor Avibactam has been introduced and shown outstanding inhibitory activity against Class A, Class C ,and Class D SBLs[73], it is still poorly effective against Class B MBLs[74]. In fact, currently, no β-lactamase inhibitor is available in clinic for Class B MBLs[20], along with the increasing number of MBL producing pathogens spreading globally[75], making them the most troublesome β-lactamases.

In addition, antibiotic potentiators that are effective against PBP2a-producing bacteria remain relatively unexplored. PBP2a is critical for the broad-spectrum β-lactam resistance in MRSA. The inhibitions in either the function or the synthesis of PBP2a are believed to be promising solutions in overcoming the resistance of MRSA[76].

To better combat the spread of antibiotic resistance, discovering more efficient and diverse antibiotic potentiators is necessary. One large group of antibiotic potentiators is the secondary metabolites of plants[21]. Due to their higher molecular complexity and enhanced scaffold diversity, they often possess inhibitory properties toward more than one resistance mechanism[77]. It is rarer for bacteria to develop resistance against multiple antimicrobial pathways at the same time. Thus the natural antibiotic potentiators are believed to have high effectiveness for suppressing the spread of antimicrobial resistance[78]. Literature has shown that many secondary metabolites of plants, especially phenolic compounds, actively potentiate antibiotics. For example, tellimagrandin I extracted from petals of *Rosa canina L.* (rose red) was found to enhance the potency of β-lactam antibiotics in MRSA[79]. The ethanolic extract of *Mangifera indica* has been demonstrated to reduce the minimum inhibitory concentrations (MICs) of tetracycline and erythromycin by 4-fold against *Staphylococcus aureus*[80]. The methanolic extract of *Tectonagrandis* combined with tetracycline has shown to cause a 2-fold reduction and 4-fold reduction on the MICs against *S. typhimurium* and *K. pneumoniae* strains, respectively[81].

1.7 Cranberry-Derived Proanthocyanidins (cPAC) As an Antibiotic Potentiator

Traditionally, the fruit of the American cranberry (*Vaccinium macrocarpon* L.) has been used as a folk medicine for the treatment of urinary tract infections[82]. Cranberry-derived proanthocyanidins (cPAC) are condensed tannins that have been demonstrated *in vitro* to hold several important antimicrobial properties against common uropathogens such as *P. aeruginosa*, *E. coli*, and *P. mirabilis*. For example, cPAC was found to impair the motilities of *P. aeruginosa*[83], *E. coli*[84], and *P. mirabilis*[85] without directly kill the bacteria. cPAC could also inhibit the quorum sensing in *P. aeruginosa*, leading to decreased virulence of the pathogen[86].

Our laboratory has recently investigated the potentiation effects of cPAC for different antibiotics (trimethoprim; sulfamethoxazole; fosfomycin; nitrofurantoin; gentamicin; kanamycin; tetracycline; azithromycin) against the common Gram-negative pathogens *E. coli*, *P. mirabilis*, and *P. aeruginosa*. These pathogens possess two resistance mechanisms that can complicate the treatment: the intrinsic antibiotic resistance, and the formation of antibiotic-tolerant biofilms. The study showed that cPAC significantly potentiated a wide range of antibiotics (Figure 2) against *E. coli*, *P. mirabilis*, and *P. aeruginosa* by depriving both antibiotic resistance mechanisms mentioned above[90]. Using 1-N-phenylnapthylamine as an indicator, it was found that bacteria's outer membranes became more permeable under the treatment of cPAC. Meanwhile, with the use of ethidium bromide (EtBr) as a fluorescent indicator, cPAC were found to act as an efflux pump inhibitor, as cells failed to pump EtBr out of their cytoplasm when treated with cPAC. The study also showed that cPAC were able to significantly potentiate sulfamethoxazole (SMX) antibiotic to eradicate persister cells, leading to much higher potency against biofilm formation of *E. coli*, *P. mirabilis*, and *P. aeruginosa*. As quorum sensing is required for normal biofilm formation[91], this effect may be linked with cPAC' ability to disturb the bacterial communication system.

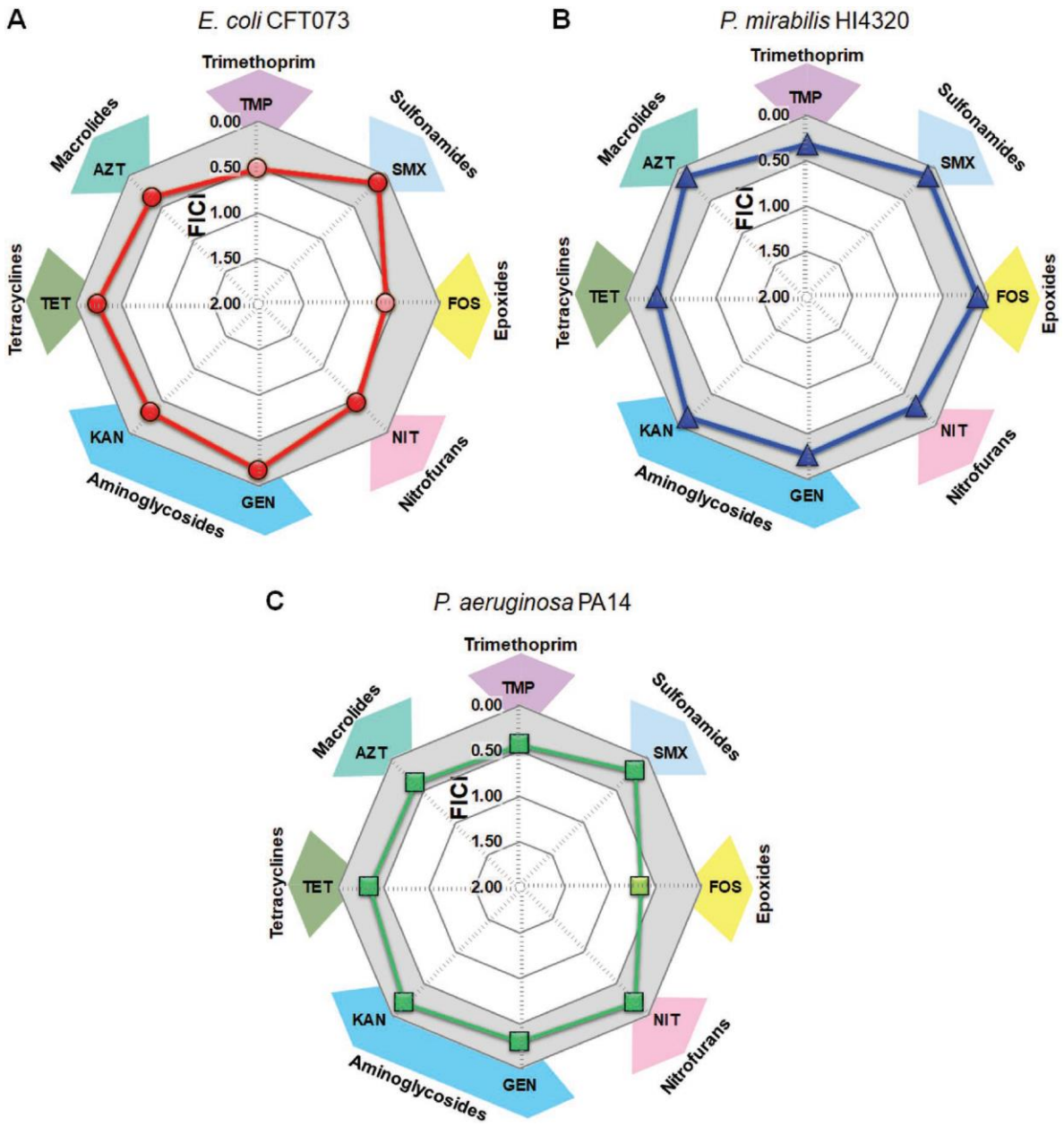

Figure 2[1]**.** The potentiating interactions of cPAC with antibiotics. The data points located in the grey shaded areas indicate synergy. TMP: trimethoprim; SMX: sulfamethoxazole; FOS: fosfomycin; NIT: nitrofurantoin; GEN: gentamicin; KAN: kanamycin; TET: tetracycline; AZT: azithromycin. A fractional inhibitory concentration index (FICI) value lower than 0.5 (highlighted in grey) indicates potentiation. Figure adapted from ref[90]

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1.8 Objectives

The successfully demonstrated antimicrobial properties and the potentiation of antibiotics motivate the need to investigate the potential effect of cPAC on the most widely used antibiotic class – β-lactam antibiotics. In fact, preliminary data acquired by Dr. Vimal Maisuria has shown that cPAC has the potential to overcome β-lactam resistance in MRSA. Therefore, it is of great interest to delve into cPAC's ability as an antimicrobial potentiator against β-lactam resistant pathogens. Specifically, this thesis will address the following aspects:

Objective 1: Investigate the effect of cPAC on common clinically important β-lactamases.

Objective 2: Explore the effects of cPAC on the variant transpeptidase PBP2a produced by methicillin-resistant *Staphylococci*.

2 Cranberry-Derived Proanthocyanidins Potentiate β-Lactam Antibiotics Against Resistant Bacteria

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2.1 ABSTRACT

The emergence and spread of extended-spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs) or variant low affinity penicillin-binding proteins (PBPs) pose a major threat to our ability to treat bacterial infection using β-lactam antibiotics. Although combinations of β-lactamase inhibitors with β-lactam agents have been clinically successful, there are no MBL inhibitors in current therapeutic use. Furthermore, recent clinical use of new generation cephalosporins targeting PBP2a, an altered PBP, has led to the emergence of resistance to these antimicrobial agents. Previous work shows that natural polyphenols such as cranberry-extracted proanthocyanidins (cPAC) can potentiate non-β-lactam antibiotics against Gram-negative bacteria. This study extends beyond previous work by investigating the *in vitro* effect of cPAC in overcoming ESBL-, MBL- and PBP2a-mediated β-lactam resistance. The results show that cPAC exhibits variable potentiation of different β-lactams against β-lactam resistant *Enterobacteriaceae* clinical isolates as well as ESBL- and MBL-producing *E. coli*. We also discovered that cPAC has broad-spectrum

inhibitory properties *in vitro* on the activity of different classes of β-lactamases, including CTX-M3 ESBL and IMP-1 MBL. Furthermore, we observe that cPAC selectively potentiates oxacillin and carbenicillin against methicillin-resistant but not methicillin-sensitive *Staphylococci*, suggesting that cPAC also interferes with PBP2a-mediated resistance. This study motivates the need for future work to identify the most bioactive compounds in cPAC and to evaluate their antibiotic potentiating efficacy *in vivo*.

2.2 IMPORTANCE

Emergence of β-lactam resistant *Enterobacteriaceae* and *Staphylococci* compromised the effectiveness of β-lactams-based therapy. By acquisition of ESBLs, MBLs or PBPs, it is highly likely that bacteria become completely resistant to the most effective β-lactam agents in the near future. In this study, we described a natural extract rich in proanthocyanidins which exerts adjuvant properties by interfering with two different resistance mechanisms. By their broad-spectrum inhibitory ability, cranberry-extracted proanthocyanidins could have the potential to enhance effectiveness of existing β-lactam agents.

2.3 INTRODUCTION

As one of the most commonly used classes of antibiotics[92], β-lactams play an important role in treating many common bacterial infections, including pneumonia, endocarditis, skin and soft tissue infection[93,94]. Resistance, however, to these essential drugs has increased over the last few decades[95–97]. Notorious examples are *Enterobacteriaceae*[98] and *Staphylococcus* species[99] that can disseminate their β-lactam resistance genes through horizontal gene transfer[100]. Resistance to β-lactam antibiotics is associated with two acquired resistance mechanisms, namely, (i) the production of β-lactamases and (ii) the synthesis of low affinity variant penicillin-binding proteins (PBPs), and also involves intrinsic resistance mechanisms, namely drug efflux and low membrane permeability. Infections with these resistant strains can complicate their treatments[101–103], by requiring higher doses or alternative antibiotics associated with greater side-effects and significant cost, and can even be associated with increased mortality[104–107]. It is thus important to counter β-lactam resistance mechanisms to improve the effectiveness of existing β-lactam antibiotics against resistant bacteria.

β-lactamases have traditionally been classified into four classes: class A to class D[47]. While classes A, C, and D all contain active-site serine and are thus termed serine-β-lactamases (SBLs)[108], class B β-lactamases utilize zinc ions in their active-site and are therefore named metallo-β-lactamases (MBLs)[109]. Another classification scheme known as the Bush-Jacoby, classifies enzymes according to their substrate and inhibitor profiles[110,111]. New generations of β-lactam antibiotics, such as extended-spectrum

cephalosporins, carbapenems, and monobactams, have been developed to be more resistant to β-lactamase hydrolysis[112,113]. Nonetheless, extended-spectrum β-lactamases (ESBLs) quickly emerged in *Enterobacteriaceae* species[98,114,115], leading to the spread of strains resistant to frequently used 3rd generation cephalosporins[112], such as cefotaxime[113]. Carbapenems, considered as "the β-lactams of last resort"[116,117], were developed to treat ESBL-producing *Enterobacteriaceae* infection[113,118], but resistance soon developed and carbapenems became less effective due to the emergence of carbapenemases[119,120]. Carbapenemases are the leading cause of CRE (carbapenem-resistant *Enterobacteriaceae*) infections[119]. Countering ESBL- and carbapenemase-producing bacteria is critical in the battle against β-lactam resistance. β-lactamase inhibitors, such as avibactam[121,122], are the most successful adjuvants used in the clinic[123,124], but their activity is limited to SBLs. Effective inhibitors for MBLs are still very scarce[125–128]. Thus, identifying molecules that can inhibit MBLs is of interest.

Another β-lactam resistance mechanism is acquisition of variant PBPs such as PBP2a in methicillin-resistant *Staphylococcus aureus* (MRSA). Variant PBPs are structurally altered with a reduced affinity to β-lactam antibiotics[129,130], and bacteria harbouring genes encoding these variants can readily survive exposure to β-lactam antibiotics. PBP2a, encoded by the *mecA* gene, is responsible for the resistance of MRSA to β-lactams (such as methicillin or oxacillin)[130]. Although cephalosporins that possess a high affinity for PBP2a[130–132] have been recently clinically approved to treat MRSA, resistance to these compounds has also emerged[130,133]. Effective treatment for MRSA infections thus remains a challenge, and adjuvants that target variant PBP2a-mediated resistance remain relatively unexplored.

Natural phenolic compounds have shown intriguing antibiotic potentiating activity *in vitro* that motivate the need for further research[134,135]. Cranberry-extracted proanthocyanidins (cPAC) consist of condensed tannins derived from the American cranberry fruit (*Vaccinium macrocarpon* L.) that possess multiple antibacterial activities of interest, such as disrupting bacterial attachment to cellular or biomaterial surfaces[136–139], reducing bacterial motility[140–145], inducing a state of iron limitation[146], and inhibiting quorum sensing[147]. cPAC also potentiate different non-β-lactam antibiotics *in vitro* against selected Gram-negative pathogens by increasing outer membrane permeability and impairing efflux pumps[148]. Yet, the ability of an extract rich in cPAC to potentiate β-lactam antibiotics against resistant bacteria or Gram-positive bacteria has not been explored.

In this *in vitro* study, we examined the effect of an extract rich in cPAC on β-lactam-resistant Gram-positive and Gram-negative bacterial strains in combination with different classes of β-lactam antibiotics,

namely penicillin derivatives, cephalosporins and carbapenems. We found that cPAC potentiate β-lactam antibiotics against several pathogenic β-lactam-resistant *Enterobacteriaceae*, and that cPAC inhibit a wide range of β-lactamases, including ESBLs and MBLs. We also observed that cPAC restore the activity of oxacillin and carbenicillin against MRSA and can overcome PBP2a-mediated resistance.

2.4 MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used are listed in Table 1 and Table 2, respectively. Gram-negative bacteria were grown in Lysogeny Broth (LB, Fisher Scientific) at 37°C with shaking (250 rpm). *Escherichia coli* strain NEB-5α and *dam*$^{-}$*/dcm*$^{-}$ *deficient E. coli (New England Biolabs Ltd)* were used for molecular cloning. *S. aureus* strains were grown in Tryptic Soy Broth (TSB, Fisher Scientific) medium at 37°C with shaking (250 rpm). Media were supplemented with antibiotics as appropriate for selection: ampicillin (AMP) 100 µg/mL (Gram-negative bacteria); kanamycin (KAN) 50 µg/mL (*E. coli*); tetracycline (TET) 10 µg/mL (*S. aureus*); chloramphenicol (CHL) 10 µg/mL (*S. aureus*).

Cranberry Proanthocyanidin

The cranberry-derived proanthocyanidins (cPAC, enriched approximately 93% proanthocyanidins) were obtained from Ocean Spray Cranberries Inc. The supplier prepared the sample according to well-established methods [139] by enriching from cranberry fruit juice extract. A dry powder of cPAC was solubilized in pure ethanol (stock solution, 20 mg/mL) and filtered (0.22 µm polyvinylidene fluoride membrane filter). The ethanol content introduced with cPAC was accounted for in all assays reported herein.

β-lactamase activity assay

Cell lysates containing β-lactamase were generated as described by Paterson *et al* [149]. Briefly, cell pellets from 5 mL overnight β-lactamase-producing *E. coli* cultures were resuspended in 500 µL of Tris Buffer (50 mM Tris–HCl pH 7.4, 160 µg/mL lysozyme), incubated at room temperature for 30 min with shaking (180 rpm), and then centrifuged to remove cell debris. The supernatant of the cell lysates was used for the nitrocefin assay.

The β-lactamase activity was measured using the nitrocefin assay as described by Viswanatha *et al*. [150]. Briefly, fresh 200 µM nitrocefin solution was prepared in 50 mM 4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid (HEPES) solution (pH 7.3) supplemented with 100 µM $ZnCl_2$ and 100 µg/mL bovine serum albumin (BSA) for class B β-lactamases. 160 µL nitrocefin solution was added to 20 µL cPAC or commercial β-lactamase inhibitor, and serially diluted in 90 µL nitrocefin solution to achieve various cPAC or inhibitor concentrations. 10 µL β-lactamase-containing cell lysates (optimal hydrolysis rate of nitrocefin was previously determined) were then added to initiate the reaction. For the experiments with zinc supplementation, 2.5 mM to 15 mM of $ZnCl_2$ was added into the nitrocefin working solution (in addition to the $ZnCl_2$ 100 µM required for the optimal MBL activity) following pre-treatment with 50 mM EDTA or 100 µg/mL cPAC. To account for the ethanol content introduced with cPAC, all wells were adjusted to contain 2 v/v % ethanol. The β-lactamase activity was measured by absorbance at 490 nm (Tecan Infinite M200 Pro microplate reader) for 10 min at room temperature and calculated as the initial slopes of absorbance plots. The residual activity was calculated as the ratio of the β-lactamase activity treated by cPAC/commercial β-lactamase inhibitor to the control β-lactamase activity.

Heterologous expression of *S. aureus blaZ* gene in *E. coli*

The *blaZ* gene encoding *S. aureus* PC1 β-lactamase was amplified by PCR from the MRSA USA300 strain using the primers PC1-*Nco*I-F/PC1-*Xho*I-R (Table 2) and following PCR conditions (annealing 53°C, extension time 40 s, 35 cycles). The amplified *blaZ* fragment was ligated into the *Nco*I and *Xho*I sites of digested pGDP1, downstream the pGDP1 P_{bla} promoter, to generate the pGDP1-bla_{PC1} construct and introduced into *E. coli* BW25113Δ*bamB*Δ*tolC* using standard cloning techniques.

Heterologous expression of *mecA* in MSSA strain

The *mecA* gene encoding PBP2a and its native promoter were amplified by PCR from the *MRSA USA300 strain* using the primers *mecA-BamH*I-F/*mecA-Sal*I-R (Table 2) and following PCR conditions (annealing 55°C, extension time 90 s, 35 cycles). The amplified *mecA* fragment was inserted into the *BamH*I and *Sal*I sites of the pJC1306 integrative vector, transformed into *E. coli* NEB-5α and selected on LB agar supplemented with ampicillin. Due to PBP2a toxicity in *E. coli*, transformants grew as tiny colonies after 24 h of incubation at 37°C and were confirmed by PCR. The observed growth defect of *E. coli* expressing *mecA* is likely due to a difference of the peptidoglycan structure between *E. coli* and *S. aureus* (in the Gram-negative, tetrapeptide chains are directly cross-linked while in the Gram-positive, tetrapeptide chains are linked by pentaglycine-cross-bridges - the site of action of PBP2a). All transformations of electrocompetent *S. aureus* strain were performed by electroporation (2.5 kV, 100Ω and 25 µF) [151] with non-methylated plasmid from *dam-*/*dcm- E. coli*. The pJC1306-*mecA* construct - verified by sequencing -

and pJC1306 empty vector control were integrated into the *S. aureus* genome at the SaPI1 *attC* site by electroporation into MSSA ATCC29213 carrying the pRN7023 integrase plasmid [152]. The *S. aureus* transformants were selected on TSA plate supplemented with chloramphenicol and tetracycline to generate MSSA-*attC*::[pJC1306-*mecA*] and MSSA-*attC*::[pJC1306] respectively.

Quantitative RT-PCR

For total RNA extraction, *S. aureus* MRSA ST241 culture were inoculated at an initial OD_{580} of 0.08 from overnight bacterial culture and grown at 37 °C with shaking in TSB medium supplemented with cPAC 25 mg/mL and/or oxacillin 1 mg/mL as indicated. To account for the ethanol content introduced with cPAC, an equivalent volume of ethanol was added for cells unexposed to cPAC. After ~4 h growth (OD_{580} ~1.5), cells were pelleted and lysed for 30 min at 37 °C with 10 mM Tris-HCl (pH 8.0), 10 mg/mL lysozyme and 0.5 mg/mL lysostaphin (Sigma-Aldrich). Total RNA was then extracted using the PureLink™ RNA Mini Kit (ThermoFisher Scientific) following the manufacturer's instructions. Remaining genomic DNA was digested with Turbo™ DNase (ThermoFisher Scientific), followed by enzyme inactivation by adding 0.01 M EDTA and heating at 75 °C for 10 min. Total cDNA was amplified with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

Each primer pair (Table 2) was verified to generate a single PCR product using MRSA ST241 DNA as template. PCR reactions (6.5 µL SYBR Green PCR Master Mix (Applied Biosystems), 0.3 µM of each forward and reverse primers, 3 µL cDNA for total volume 13 µL) were performed using the 7500 Real Time PCR System (Applied Biosystems). Relative mRNA expression was quantified using the comparative $2^{-\Delta\Delta CT}$ method with the *gyrB* mRNA in untreated MRSA as control. Transcriptomic experiments were done with 4 independent biological replicates, 2 technical replicates.

Checkerboard microdilution assay

Resazurin checkerboard assay was performed in 96-well plates as previously described [153] with an inoculum of ~10^6 CFU/mL in Cation-adjusted Mueller-Hinton broth (CAMHB, Fisher Scientific). Following incubation at 37°C for 18 h under static conditions, the Minimum Inhibitory Concentration (MIC) for cPAC or each antibiotic that inhibited bacterial growth was determined by visual observation. For each treatment, the MIC was confirmed at least once by fluorescence measurements of the reduced resorufin with excitation/emission at 530/590 nm. All checkerboard assays were done with at least 4 independent replicates. The fractional inhibitory concentration index (FICI) for each combination was

calculated by using the following formula: FICI= ($MIC_{antibiotic,\ in\ combination}$ /$MIC_{antibiotic,\ alone}$) + ($MIC_{cPAC,\ in\ combination}$ /$MIC_{cPAC,\ alone}$). In this study, the FIC index is interpreted as: a potentiating effect when FICI≤0.5 and an indifferent effect when 1<FICI<4[154]. Controls without cPAC were performed for each β-lactam/strain combination to confirm that reported effects were not solvent related (Fig. S1). To preserve the limited supply of cPAC, the $MIC_{cPAC,\ alone}$ was separately confirmed for each strain and not reached in all checkerboard plates. For the FICI calculation, if the $MIC_{cPAC,\ alone}$ was not reached in a checkerboard plate, it was estimated as the next highest dilution.

2.5 RESULTS

cPAC showed variable potentiation with β-lactams against ESBL-producing *Enterobacteriaceae* clinical strains

We started by testing the effect of cPAC against ESBL-producing *Enterobacteriaceae,* namely *Klebsiella* spp. and *Proteus mirabilis* clinical strains due to their clinical importance and well-known high β-lactam resistance[155,156]. As ESBLs degrade a broad range of β-lactams, including 3rd generation cephalosporins[111,113], we examined the potentiation effect of cPAC in combination with cefotaxime as well as ampicillin, a penicillin-type β-lactam. Checkerboard assays using cPAC in combination with β-lactams were performed to determine the potentiation effects against *Klebsiella* spp. and *P. mirabilis* clinical isolates producing different β-lactamases, including ESBLs (Figure 3A). The *Klebsiella* spp. and *P. mirabilis* clinical isolates show β-lactam resistance of varying degree, with MIC of ampicillin and cefotaxime varying from 8 to >10000 µg/mL (Table S1). cPAC showed potentiation with cefotaxime, with FICI values ≤0.5, against CTX-M cephalosporinase-producing *Klebsiella* sp. and *P. mirabilis* strains, while no potentiation was observed against a cefotaxime-hydrolysing SHV-27-producing *Klebsiella pneumoniae* strain. For instance, 600 µg/mL cPAC potentiated cefotaxime with a reduction in MIC of up to 64-fold against CTX-M15-producing *Klebsiella* sp. (Figure 3B, Table S1). cPAC also showed potentiating effect with ampicillin, but only against the CTX-M/OXA-producing *P. mirabilis* strain and neither of the *Klebsiella* strains (Figure 3A, Table S1). These results indicate that the potentiation activity of cPAC against ESBL-producing *Enterobacteriaceae* is variable depending on the β-lactam drug, the β-lactamase expressed and/or bacterial strain.

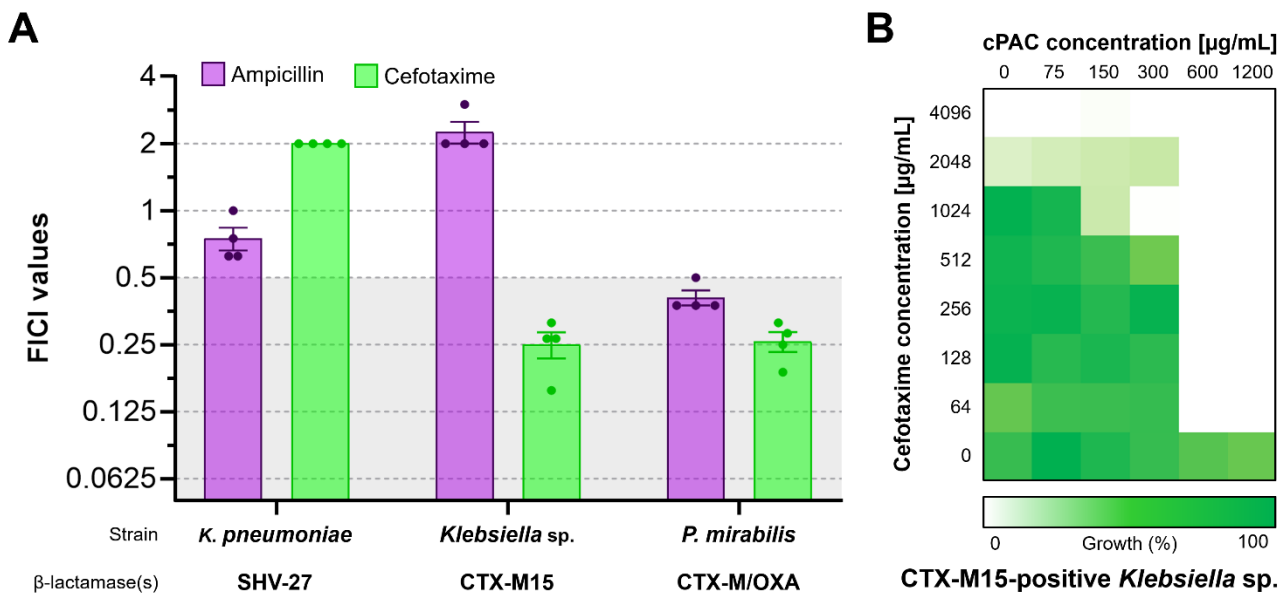

Figure 3. Effect of cPAC with cefotaxime and ampicillin on the growth of ESBL-producing *Enterobacteriaceae*. (A) FICI values for cPAC combinations with ampicillin or cefotaxime were calculated from checkerboard assays. *Klebsiella* sp. and *Proteus mirabilis* clinical strains produced various β-lactamases SHV-27, CTX-M15, CTX-M and OXA as indicated. Gray shaded area represents a FICI ≤0.5. Bars represent the average of four independent replicates and the error bars show the SEM. (B) Representative checkerboard assay showing the effect of cPAC in combination with cefotaxime on the growth of CTX-M15-positive *Klebsiella* sp. Percentage of bacterial growth inhibition was determined by measuring the fluorescence of resorufin.

cPAC potentiated the activity of β-lactams against *E. coli* producing multiple classes of serine β-lactamases, including ESBLs

Although cPAC was found to potentiate some β-lactam antibiotics against ESBL-producing *Klebsiella* and *P. mirabilis* clinical isolates, the strains' diverse genetic backgrounds prevented us from clearly characterizing the adjuvant activity of cPAC. To better understand the potentiating effect of cPAC against bacteria producing different β-lactamases, we used a genetically defined *E. coli* BW25113Δ*bamB*Δ*tolC* strain, which is a hyperpermeable efflux pump-deficient *E. coli* strain developed by Cox *et al.* as part of the antibiotic resistance platform[157] that can strongly express various β-lactamases under the control of the P_{bla} promoter. Importantly, the effect of cPAC on β-lactamase-mediated resistance can be investigated in the *bamB*/*tolC* deficient *E. coli* strain in isolation of cPAC's influence on membrane permeability and antibiotic efflux, effects which were previously described[148]. We selected a range of clinically relevant

SBLs that lead to β-lactam MIC levels from 512 µg/mL to >10000 for ampicillin and from 32 to 4096 µg/mL for cefotaxime (Table S2).

Checkerboard assays were performed, as shown in Figure 4 using cPAC in combination with various β-lactam antibiotics, chosen according to the distinctive substrates of the selected SBLs, namely ampicillin for penicillinase- or cefotaxime for cephalosporinase-producing *E. coli*, according to the classification proposed by Bush and Jacoby[110,111]. We found that cPAC potentiated cefotaxime against class A CTX-M-type ESBLs but not the class C AmpC cephalosporinase ACT-28 (Figure 4A). Antibiotic potentiation was observed against *E. coli* producing class A TEM-30 (also known as IRT-2, for inhibitor-resistant TEM 2), while the interaction between cPAC and ampicillin showed an effect with FICI close to 0.5 against class A TEM-1 penicillinase. No potentiation effect was observed against *E. coli* producing class A PC1 penicillinase.

To explore whether cPAC overcame β-lactamase-mediated resistance by inhibiting β-lactamase activity, we tested the effect of cPAC on *in vitro* β-lactamase activity using the nitrocefin assay with cell-free lysates generated from SBL-expressing *E. coli* strains. We used as a control avibactam, a new-generation β-lactamase inhibitor with potent activity against many class A SBLs[73,122]. Figure 4C shows that cPAC inhibited the selected β-lactamases in a dose-dependent manner, with half maximal inhibitory concentrations (IC_{50}) at approximately 50 µg/mL for both SBLs, similar to the effect of about 4 ng/mL avibactam. Taken together, we observe that the efficacy of cPAC in combination with ampicillin or cefotaxime depends on the β-lactamase, and cPAC can inhibit different classes of β-lactamases in a dose-dependent manner.

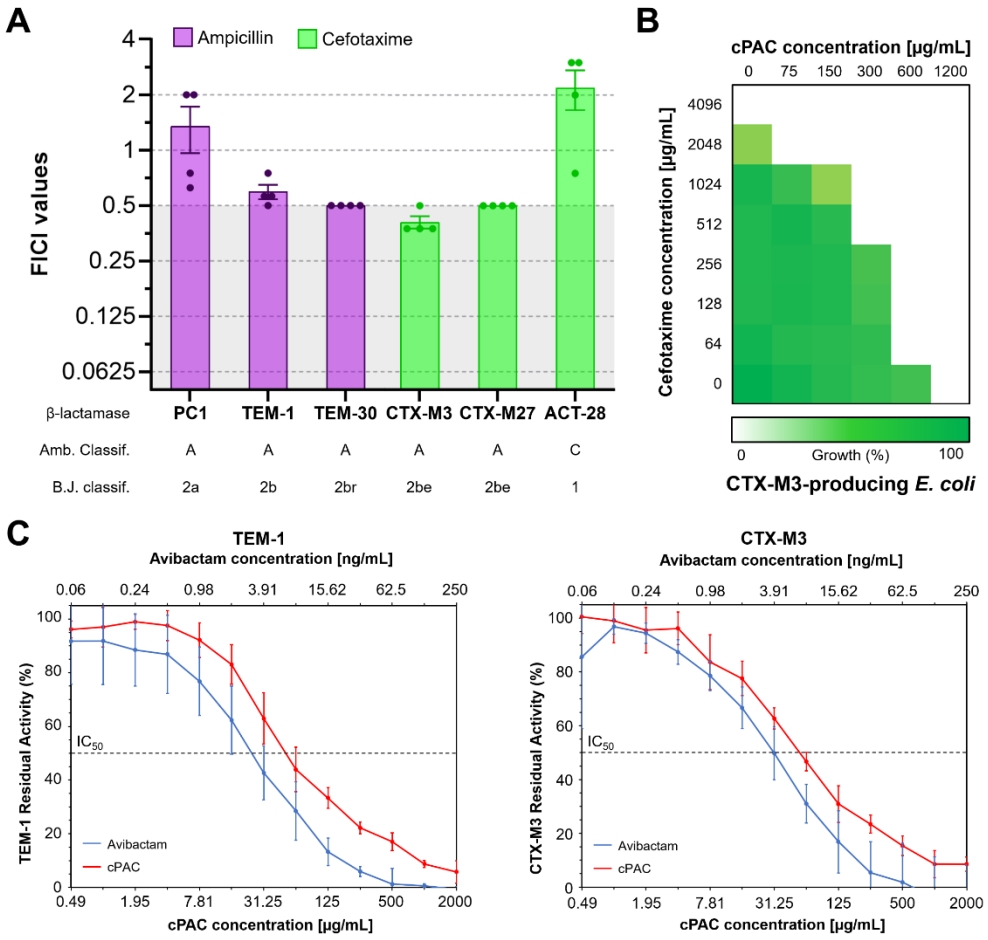

Figure 4. Effect of cPAC with β-lactams on the growth of serine-β-lactamase producing *E. coli* and inhibition of β-lactamase activity *in vitro*. Checkerboard assays using the Δ*bamB*Δ*tolC E. coli* strain expressing different SBLs (as indicated) were done for the combination of cPAC with ampicillin or cefotaxime against penicillinase- and cephalosporinase-producing *E. coli*, respectively. (A) Gray shaded area represents a FICI ≤0.5. Bars represent the average of four independent replicates and the error bars show the SEM. Amb. classif.: Ambler classification, B.J. classif.: Bush-Jacoby classification. (B) Representative checkerboard assay showing the effect of cPAC in combination with cefotaxime on the growth of CTX-M3-producing *E. coli*. Percentage of bacterial growth inhibition was determined by measuring the fluorescence of resorufin. (C) Dose-dependent *in vitro* inhibitory effect of cPAC and avibactam on TEM-1 penicillinase and CTX-M3 ESBL. IC_{50}: half maximal inhibitory concentration. Data points represent the average of six replicates in two independent experiments and the error bars show the SD.

cPAC acts as a strong inhibitor of MBLs without sequestering the zinc co-factor

MBLs, which hydrolyze carbapenems, represent a clinical challenge as none of the currently used β-lactamase inhibitors are effective at inhibiting their hydrolytic activity[158]. We investigated the potentiating

effect of cPAC with meropenem, a commonly used broad-spectrum carbapenem, against representative MBLs. Results of the checkerboard assays suggest that cPAC potentiated meropenem against MBL-producing *E. coli* including IMP-1 (Figure 5A and Figure 5B). In particular, strong potentiation was observed against *E. coli* expressing Sfh-1 enzyme, whereby 75 µg/mL cPAC reversed meropenem resistance and reduced its MIC to 2 µg/mL (Figure S1, Table S2).

To better understand how cPAC potentiates meropenem to overcome MBL-mediated resistance, we tested the effect of cPAC on the *in vitro* β-lactamase activity of IMP-1-containing cell-free lysates using the nitrocefin assay, and compared it to captopril, a thiol-containing small molecule inhibitor of several MBLs[159]. Approximately 27 µg/mL captopril or 93 µg/mL cPAC were required to achieve 50% inhibition of IMP-1 activity (Figure 5C), suggesting that cPAC can act as a MBL inhibitor. Since MBLs require zinc as co-factor, zinc chelating ability is a common mechanism of MBL inhibition[127,160,161], and EDTA, a strong zinc chelator[162] inhibits MBLs[163]. We therefore asked whether cPAC inhibition of IMP-1 was zinc-dependent by testing its effect on the *in vitro* IMP-1 β-lactamase activity using cell-free lysates. Treatment of IMP-1-containing lysates with either 50 mM EDTA or 100 µg/mL cPAC (Figure 5D) reduced the IMP-1 β-lactamase activity by approximately 46% in both cases. Supplementation with $ZnCl_2$ restored IMP-1 activity following pre-treatment with EDTA, confirming that loss of IMP-1 activity was attributable to the unavailability of zinc ions. However, following pre-treatment with cPAC, IMP-1 activity remained impaired upon supplementation with $ZnCl_2$ to concentrations up to 15 mM. The result suggests different mechanisms of IMP-1 inhibition by cPAC, rather than zinc co-factor binding.

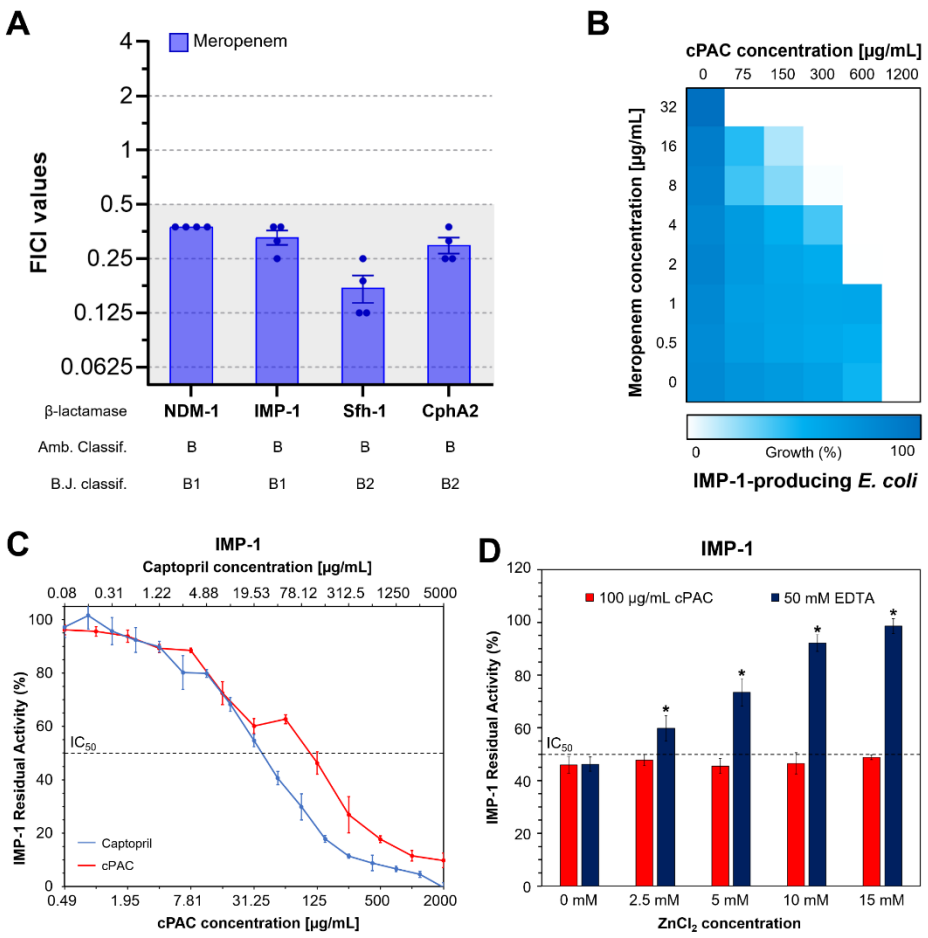

Figure 5. Effect of cPAC with meropenem on the growth of metallo-β-lactamase producing *E. coli* and inhibition of metallo β-lactamase activity *in vitro*. (A) Checkerboard assays using the Δ*bamB*Δ*tolC* *E. coli* strain expressing different MBLs were done for the combination of cPAC with meropenem. Gray shaded area represents a FICI ≤0.5. Bars represent the average of four independent replicates and the error bars show the SEM. Amb. classif.: Ambler classification, B.J. classif.: Bush-Jacoby classification. (B) Representative checkerboard assays showing the effect of cPAC in combination with meropenem on the growth of IMP-1-producing *E. coli*. Percentage of bacterial growth inhibition was determined by measuring the fluorescence of resorufin. (C) Dose-dependent *in vitro* inhibitory effect of cPAC and captopril on IMP-1. IC_{50}: half maximal inhibitory concentration. (D) IMP-1 β-lactamase residual activity at different concentrations of $ZnCl_2$, first treated with 50 mM EDTA or with 100 µg/mL cPAC. Data points represent the average of six replicates in two independent experiments and the error bars show the SD. * $P < 0.005$ compared to control without $ZnCl_2$ treatment

Potentiating effects of cPAC against methicillin-resistant *Staphylococcus* spp.

Methicillin-resistant *Staphylococcus* spp. are widespread *β-lactam* resistant Gram-positive bacteria that express the variant PBP2a[11], a key determinant of resistance to β-lactams. A cranberry extract which

contains proanthocyanidins has been described to have antibacterial properties against *S. aureus*, including MRSA[164]. It has also been shown that a cranberry press cake extract potentiates β-lactams against MRSA[165]. This led us to ask whether a proanthocyanidin enriched extract, namely cPAC, in addition to inhibition of β-lactamases, could also mitigate other mechanisms of β-lactam resistance. Therefore, we conducted checkerboard assays to investigate the effects of cPAC in combination with oxacillin and carbenicillin on the growth of ten methicillin-resistant *S. aureus* (MRSA*)* clinical isolates[166], two methicillin-sensitive *S. aureus* (MSSA) reference strains, one methicillin-resistant *S. epidermidis* (MRSE) clinical isolate and one methicillin-sensitive *S. epidermidis* MSSE clinical isolate. Interestingly, cPAC strongly potentiated carbenicillin and oxacillin to inhibit the growth of all methicillin-resistant *Staphylococci* strains (ten MRSA and one MRSE) with FICI ranging from 0.16 to 0.5, but failed to potentiate either β-lactam antibiotics against methicillin-sensitive *Staphylococci* strains (two MSSA and one MSSE) (Figure 6A, Figure 6B and Table S3). The MICs and FICI values are summarized in Table S3. MIC of oxacillin and carbenicillin for MRSA clinical isolates varied from 4 to 2048 μg/mL, and the combination of cPAC with oxacillin or carbenicillin resulted in an 8 to 64-fold reduction in MIC. We also tested the common non-β-lactam antibiotics used to treat *S. aureus* infection, namely daptomycin (lipopeptide), linezolid (oxazolidinone), vancomycin (glycopeptide) and ciprofloxacin (fluoroquinolone) [167,168], and generally found no potentiating interactions with cPAC for both tested methicillin-resistant and methicillin-susceptible *Staphylococci* strains (Figure S2).

Since our results indicate that cPAC potentiate β-lactam antibiotics in MRSA/MRSE but not MSSA/MSSE strains, this raised the possibility that cPAC may target a PBP2a-mediated resistance mechanism. To examine this hypothesis, we integrated a plasmid-borne *mecA* gene encoding PBP2a whose expression is under the control of its native promoter within the chromosome of MSSA reference strain ATCC 29213 (MSSA-*attC*::[pJC1306-*mecA*]) to generate an isogenic MRSA construct. Checkerboard assays were then performed to compare this *mecA*-expressing construct to its isogenic empty vector control. cPAC potentiated oxacillin and carbenicillin in the *mecA*-expressing strain, with a reduction of their respective MICs from 64 to 8 μg/mL and 128 to 16 μg/mL with 37.5 μg/mL cPAC (Figure 6C and Table S3). We also evaluated the expression of *mecA* in MRSA ST241 after exposure to cPAC and/or oxacillin (Figure S3). The results suggest that the effect of cPAC on *mecA* mediated resistance is unlikely to be fully attributable to a perturbation of *mecA* gene expression. Taken together, these data strongly suggest that cPAC interferes with the *mecA* mediated PBP2a-associated resistance pathway, however the precise mechanism of action remains unknown.

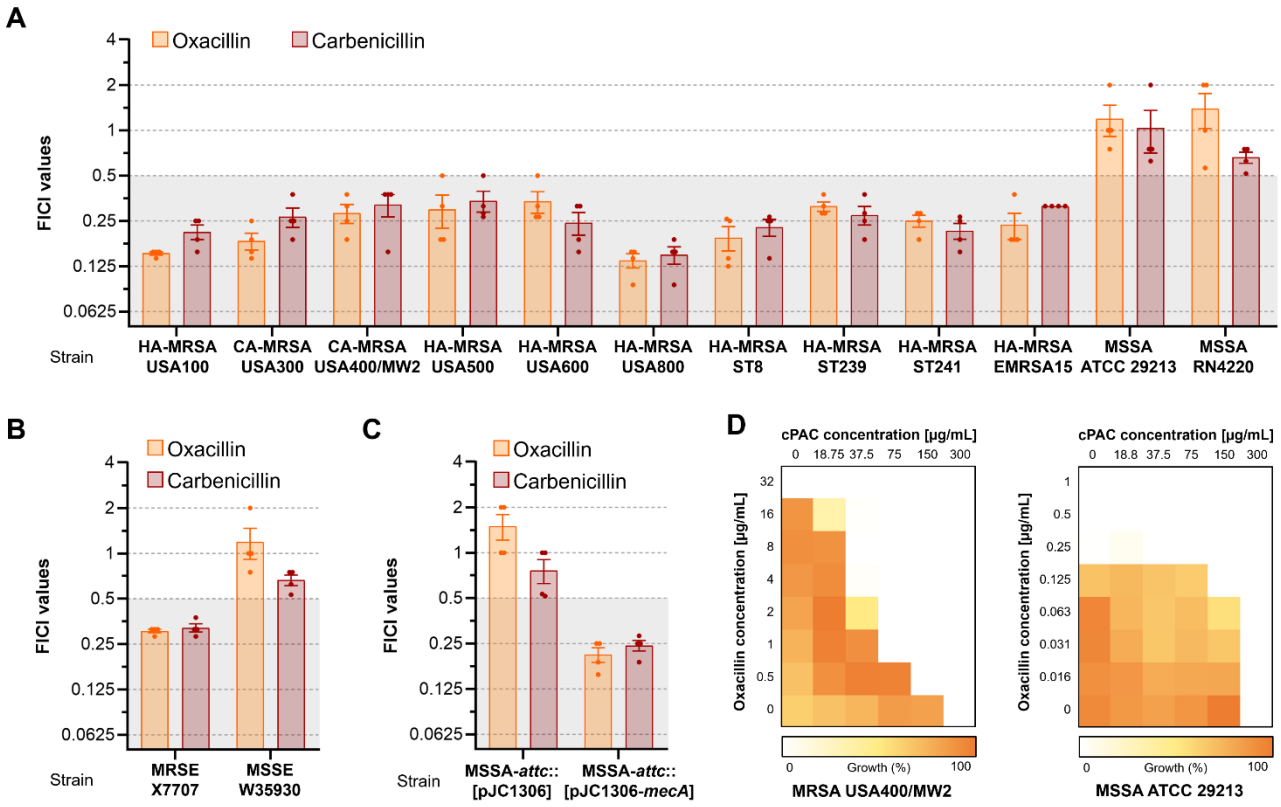

Figure 6. Effect of cPAC with penicillin-derivatives on the growth of methicillin-resistant *Staphylococci* strains. Checkerboard assays were done for the combination of cPAC with oxacillin and carbenicillin to determine the FICI for each combination in (A) *S. aureus* clinical strains, (B) *S. epidermidis* and (C) genetically modified *S. aureus* strains. Gray shaded area represents a FICI ≤0.5. Bars represent the average of four independent replicates and the error bars show the SEM. (D) Representative checkerboard assay showing the effect of cPAC in combination with oxacillin on the growth of MRSA USA400/MW2 and MSSA ATCC 29213. Percentage of bacterial growth inhibition was determined by measuring the fluorescence of resorufin.

3 DISCUSSION

In this *in vitro* study, we report for the first time that cPAC in combination with ampicillin and cefotaxime inhibits the growth of ESBL-producing pathogens and overcomes MBL-mediated carbapenem resistance when combined with meropenem. Our results thus motivate the need for future *in vivo* studies to explore the potential use of cPAC or its most bioactive compounds in decreasing the amount of antibiotic required to treat infections caused by ESBL- and carbapenemase-producing pathogens. We also report that cPAC inhibits the growth of methicillin-resistant *Staphylococcus* spp. when combined with penicillin-type β-lactams by interfering with PBP2a-mediated resistance.

β-lactam antibiotics remain the most common treatment for *Enterobacteriaceae*-associated infections, which include infections in the central nervous system, lower respiratory tract, bloodstream, gastrointestinal and urinary tracts[169,170]. However, the acquisition of resistance through the production of ESBLs complicates antibiotic therapy and can lead to treatment failure[171]. It has been described that the polyphenol epigallocatechin gallate (EGCg) restores the activity of cefotaxime[172] and aztreonam, a monobactam β-lactam[173], against ESBL-producing *E. coli* and *P. aeruginosa*, respectively. Furthermore, it has been shown that EGCg can potentiate several non-β-lactam antibiotics against ESBL- and carbapenemase-producing *K. pneumoniae* clinical isolates by inhibiting drug efflux[174]. Our discovery that cPAC affects the MIC of cefotaxime for ESBL-producing *K. pneumoniae* and *P. mirabilis* strains raises the promising possibility of expanding the activity of existing β-lactam antibiotics to ESBL-producing *Enterobacteriaceae,* and our results highlight the need to further study the potential for cPAC to serve as a broad-spectrum β-lactamase inhibitor.

Recently, Cox and coworkers developed the antibiotic resistance platform (ARP)[157], a library of *E. coli* constructs that express a wide range of resistance determinants, to facilitate the discovery of new antimicrobial agents and adjuvants that target specific resistance elements. Using the ARP β-lactamase collection, we have shown that cPAC enhances the effectiveness of β-lactam antibiotics, including ampicillin, cefotaxime and meropenem, against *E. coli* that produce inhibitor-resistant penicillinase TEM-30, CTX-M-type extended-spectrum cephalosporinases and carbapenemases, respectively.

Despite the commercial development of several effective SBL inhibitors, none yet exist for MBLs. Due to the worldwide dissemination of MBL-producing strains that contribute significantly to the emergence of carbapenem-resistant bacteria[120,175–177], and the lack of inhibitors, MBLs are considered as the most problematic β-lactamases[178]. We have shown that cPAC can inhibit the activity of MBLs, including the most widespread NDM- and IMP-type enzymes[179,180]. The emerging NDM-type carbapenemases are especially problematic as they rapidly expand within the *Enterobacteriaceae* pathogens[181], conferring resistance to nearly all β-lactam antibiotics including carbapenems. Consequently, pathogens that have acquired NDM-1 are extremely difficult to treat and have rapidly spread worldwide[13], and compounds that inhibit a broad spectrum of MBLs, particularly NDM-1, would be particularly valuable in treating drug resistant infections.

To date, the development of inhibitors for MBLs has focused on the discovery or synthesis of compounds that bind and/or chelate zinc ions[127,160,161]. Although compounds, such as EDTA[163], captopril[159], certain

thiol derivatives[182] or fungus-derived molecule[183], can inhibit MBLs *in vitro*, their mechanism of inhibition presents potential drawbacks. Since the binding/chelating of zinc ions is not target-specific, these compounds can also possibly plunder zinc ions from host metalloproteins, which play essential roles in DNA replication and RNA processing pathways[184]. In addition, increases in zinc concentration can reverse the MBL inhibition and restore their activity. We have demonstrated that cPAC inhibition of MBL IMP-1 is independent of the zinc concentration, suggesting that cPAC does not act on the MBL through the removal of zinc ions, and its mechanism of action on MBLs remains to be determined. These results thus provide a starting point for investigating the mechanism of inhibition of cPAC on MBLs.

In addition to β-lactamase production, the expression of the variant transpeptidase PBP2a in *Staphylococcus* species, including *S. aureus* and *S. epidermidis*, is another major mechanism of resistance to β-lactams. Various plant-derived polyphenols have been shown to potentiate β-lactams[134,135,185]; yet, the β-lactam potentiating activity of an extract rich in cPAC has not previously been examined. A previous study reports the effect of a bulk cranberry press cake extract having a low or negligible concentration of proanthocyanidins, in combination with β-lactams against *S. aureus* strains[165]. The fraction showing the highest activity against MRSA contains polyphenol compounds which are structurally close to proanthocyanidins, such as catechin, anthocyanidins or quercetin. It has also been demonstrated that polyphenolic compounds such as EGCg, potentiate β-lactams by interfering with PBP2/PBP2a complex and thus inhibiting its activity[186], due to disruption of the membrane architecture[187]. Quinazolinone compounds potentiate piperacillin/tazobactam by causing a conformational change of PBP2a[188]. Other compounds, such as corilagin, tellimagrandin I and II[189,190] and ZP-CT-A proanthocyanidin[191], also inhibit the growth of MRSA strains when combined with β-lactams. In this study, we discovered that cPAC can significantly reduce the concentrations of oxacillin and carbenicillin required to inhibit the growth of PBP2a-producing *Staphylococcus* species, without significantly decreasing PBP2a-encoding *mecA* gene expression.

The observed interactions between cPAC and β-lactamases as well as PBP2a, which enhance the effectiveness of multiple β-lactams, warrants further investigation of their mechanisms of action. Although we did not identify the mechanisms in this study, we did perform experiments to exclude the possibility that the cranberry extract inhibits (i) MBL activity by binding the zinc co-factor (Figure 5), and (ii) *mecA* gene expression (Figure S3). We also clearly show that cPAC interferes with the *mecA* mediated PBP2a-associated resistance pathway (Figure 6). The cPAC extract is a mixture which contains

monomeric and oligomeric flavonoids and proanthocyanidins[139,192,193]. This study aimed at evaluating the effect of cPAC combined with β-lactams on a variety of β-lactamases without examining the distinct effect of each compound. A deeper understanding of the molecular interaction of purified oligomeric epicatechin units present in cPAC with β-lactamases and/or PBP2a may contribute to the development of novel compounds that target β-lactam resistance and further expand the use of existing β-lactams. While identifying the bioactive components of cPAC and their specific mechanisms of action is a complex task that was beyond the scope of this study, our results do motivate the need for such work. Further *in vivo* studies should also evaluate the antibiotic potentiating efficacy of the most bioactive compounds in cPAC to understand their potential usefulness in the treatment of drug resistant bacterial infections.

4 Conclusion and Future Work

The emergence of antibiotic resistance has become a global concern. As the most commonly used antibiotics, β-lactam antibiotics also greatly suffer from antibiotic resistance. The resistance to these ubiquitous antibiotics is especially costly as it seriously limits the options of antibiotics for treatments on bacterial infections. This thesis discovered that the natural compounds extracted from the American cranberry, cPAC, can interfere with two important β-lactam antibiotic resistance mechanisms – β-lactamases and PBP2a - thus considerably decreasing the concentrations of relevant β-lactam antibiotics required to inhibit the growth of β-lactamase producing Gram-negative bacteria and methicillin-resistant *Staphylococci* spp..

We demonstrated that cPAC effectively potentiates cefotaxime against ESBL-producing pathogens *Klebsiella* sp. and *P. mirabilis*. Using ARP β-lactamase collection, we extended the range of β-lactamases studied and discovered significant potentiation against *E. coli* strains that produce inhibitor-resistant penicillinase TEM-30, CTX-M-type extended-spectrum cephalosporinases, and carbapenemases. We also showed that cPAC significantly improves the efficacy of meropenem against MBL-mediated carbapenem-resistant pathogens, in a mechanism different from chelation of zinc ions. Through *in vitro* nitrocefin assays, we provided evidence that the potentiation can be attributed to the inhibitory effects of cPAC to the activity of β-lactamases. The fact that cPAC inhibit a broad spectrum of β-lactamases with high clinical importance demonstrates its potential value in overcoming resistance caused by β-lactamases.

In addition, by performing *in vitro* checkerboard assays, we found cPAC strongly potentiates oxacillin and carbenicillin against MRSA and MRSE, but generally fails to potentiate the same antibiotics against MSSA and MSSE. Through genetically constructed PBP2a-producing MSSA, we successfully showed

that the potentiation effects are due to the interactions between cPAC and PBP2a. While the exact mechanisms remain uncertain, the results indicate a potential of cPAC in mitigating PBP2a-associated antibiotic resistance.

As cPAC shows valuable abilities in fighting against β-lactam antibiotic resistance, further investigations can be performed to delve into its medical potential. Firstly, the experiments are so far limited to *in vitro* environment. The efficacy and safety of cPAC in mammalian and eukaryotic hosts are not completely understood. Studying the effect of cPAC in live models is necessary to simulate and understand the interactions of cPAC in the human body. *In vivo* experiments with insects and mice are therefore of interest. Secondly, cPAC is a mixture of compounds with various effects on different β-lactam resistance mechanisms. Each compound may have functions and impact on specific resistance mechanisms. Understanding the effects of purified compounds and their possible synergies/antagonisms when dealing with different β-lactam resistance mechanisms can help identify the most suitable compounds to target each specific mechanism and contribute to the development of novel drugs to overcome β-lactam resistance. Thirdly, this thesis provides evidence that cPAC interacts with PBP2a in MRSA, but the mechanisms behind it remain unknown. Exploring the mechanisms may help the discovery of innovative solutions to conquer PBP2a-mediated β-lactam resistance. Finally, many plant-derived polyphenolic compounds, such as EGCg, have been shown to be also effective against β-lactam resistance. The polyphenolic compounds in cPAC may share similar functional structures with other plant-derived compounds. After purifying the bioactive compounds from cPAC, it can be worthwhile to reveal the potentially common structures, so that more efficient medications based on such bioactive structures can be developed.

The promising results shown in this thesis demonstrate the potential of cPAC as an agent to overcome β-lactam resistance and reflect the value of plant derived compounds. Many plant derived compounds themselves may have limited antimicrobial potency. However, through purification and modification, they can become valuable antibiotic potentiators against various antibiotic resistance mechanisms. The spread of antibiotic resistance has become a significant social concern. The more options we have to combat antibiotic resistance, the more advantages we will get in this prolonged battle.

5 References

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6 Appendix

Table S1. Interactions of cPAC with ampicillin or cefotaxime for growth inhibition of Gram-negative pathogenic isolates. Data correspond to the most representative FICI values.

		β-lactam		cPAC		
Strains	Antibiotic	MIC_{alone} (µg/mL)	MIC_{comb} (µg/mL)	MIC_{alone} (µg/mL)	MIC_{comb} (µg/mL)	FICI
K. pneumoniae H0142357 (SHV-27)	AMP	512	256	2400	300	0.63
	CTX	8	8	2400	2400	2
Klebsiella sp. 824A (CTX-M15)	AMP	8192	8192	2400	2400	2
	CTX	4096	64	2400	600	0.27(*)
P. mirabilis 698B (CTX-M/OXA)	AMP	16384	2048	1600	400	0.38(*)
	CTX	2048	256	1600	200	0.25(*)

AMP: ampicillin, CTX: cefotaxime. FICI ≤0.5 is noted with an asterisk (*)

Table S2. Interactions of cPAC and antibiotics for growth inhibition of SBL- and MBL-expressing *E. coli*. Data correspond to the most representative FICI values.

			β-lactam		cPAC		
β-lactamase	Bush-Jacoby and type of β-lactamase	Antibiotic	MIC_{alone} (μg/mL)	MIC_{comb} (μg/mL)	MIC_{alone} (μg/mL)	MIC_{comb} (μg/mL)	FICI
PC-1	2a - SBL	AMP	512	512	1200	1200	2
TEM-1	2b - SBL	AMP	16384	1024	1200	600	0.56
TEM-30	2br - ESBL	AMP	2048	512	1200	300	0.5(*)
CTX-M3	2be - ESBL	CTX	4096	512	1200	300	0.38(*)
CTX-M27	2be - ESBL	CTX	4096	1024	1200	300	0.5(*)
ACT-28	1 - SBL	CTX	32	64	1200	1200	3
IMP-1	3a - MBL	MEM	64	8	1200	300	0.38(*)
NDM-1	3a - MBL	MEM	64	16	1200	150	0.38(*)
Sfh-1	3b - MBL	MEM	16	2	1200	75	0.19(*)
CphA2	3b - MBL	MEM	1024	128	1200	150	0.25(*)

AMP: ampicillin, CTX: cefotaxime, MEM: meropenem, SBL: Serine β-lactamase, ESBL: Extended Spectrum β-lactamase and MBL: Metallo β-lactamase. FICI ≤0.5 is noted with an asterisk (*)

Table S3. Interactions of cPAC and penicillin derivatives for growth inhibition of *Staphylococci* strains. Data correspond to the most representative FICI values.[a]

		β-lactam		cPAC		
Strains	Antibiotic	MIC_{alone} (μg/mL)	MIC_{comb} (μg/mL)	MIC_{alone} (μg/mL)	MIC_{comb} (μg/mL)	FICI
HA-MRSA USA100	OXA	128	4	300	37.5	0.16(*)
	CAR	128	8	300	37.5	0.19(*)
CA-MRSA USA300	OXA	32	2	300	37.5	0.19(*)
	CAR	64	8	300	37.5	0.25(*)
CA-MRSA USA400/MW2	OXA	32	4	300	37.5	0.25(*)
	CAR	128	16	300	75	0.38(*)
HA-MRSA USA500	OXA	4	0.25	300	37.5	0.19(*)
	CAR	64	4	300	75	0.31(*)
HA-MRSA USA600	OXA	512	8	300	75	0.27(*)
	CAR	2048	128	300	75	0.31(*)
HA-MRSA USA800	OXA	128	4	300	37.5	0.16(*)
	CAR	512	16	300	37.5	0.16(*)
HA-MRSA ST8	OXA	128	16	300	37.5	0.25(*)
	CAR	512	64	300	37.5	0.25(*)
HA-MRSA ST239	OXA	256	16	300	75	0.31(*)
	CAR	1024	32	300	75	0.28(*)
HA-MRSA ST241	OXA	512	16	300	75	0.28(*)
	CAR	1024	128	300	37.5	0.25(*)
HA-MRSA ERMSA15	OXA	32	2	300	37.5	0.19(*)
	CAR	64	4	300	75	0.31(*)
MSSA RN4220	OXA	0.125	0.0625	300	150	1
	CAR	1	0.25	300	150	0.75
MSSA ATCC29213	OXA	0.25	0.125	300	150	1
	CAR	4	1	300	150	0.75
	OXA	0.125	0.125	300	300	2

MSSA-*attc*::[pJC1306]	CAR	4	2	300	150	1
MSSA-*attc*::[pJC1306-*mecA*]	OXA	64	8	300	37.5	0.25(*)
	CAR	128	16	300	37.5	0.25(*)
MRSE X7707	OXA	256	8	300	75	0.28(*)
	CAR	512	32	300	75	0.31(*)
MSSE W35930	OXA	0.25	0.125	300	150	1
	CAR	4	1	300	150	0.75

OXA: oxacillin, CAR: carbenicillin. FICI ≤0.5 is noted (*)

[a] Preliminary experiments conducted by Dr. Vimal Maisuria

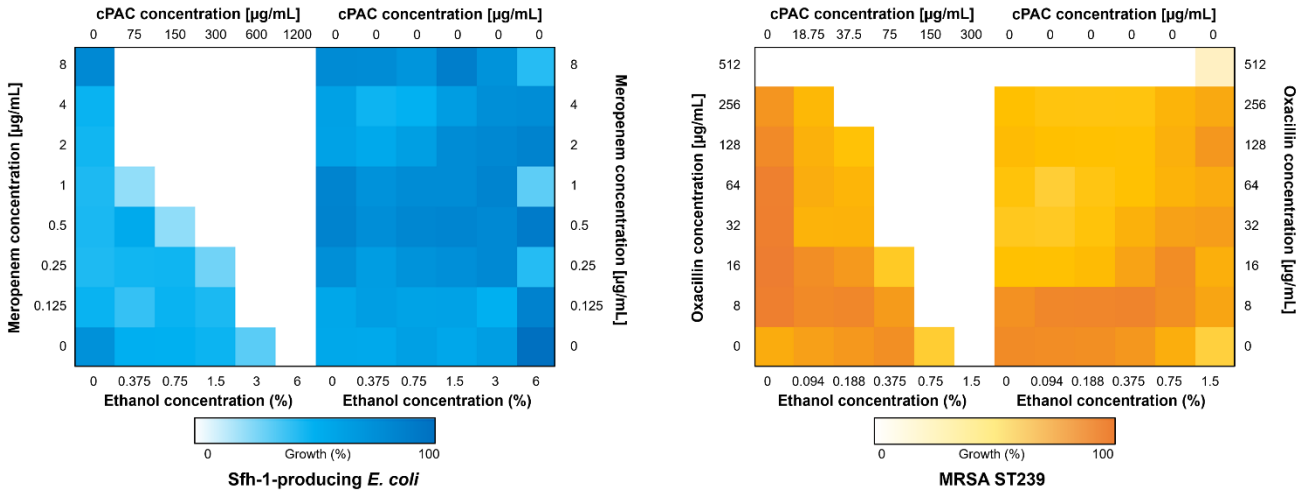

Figure S1. Representative example of a control experiment without cPAC assessed using a side-by-side checkerboard assay. Left side: microdilution assay using cPAC 20 mg/mL stock solution solubilized in ethanol; right side: microdilution assay using ethanol without cPAC in which ethanol concentration is equivalent to the left side.

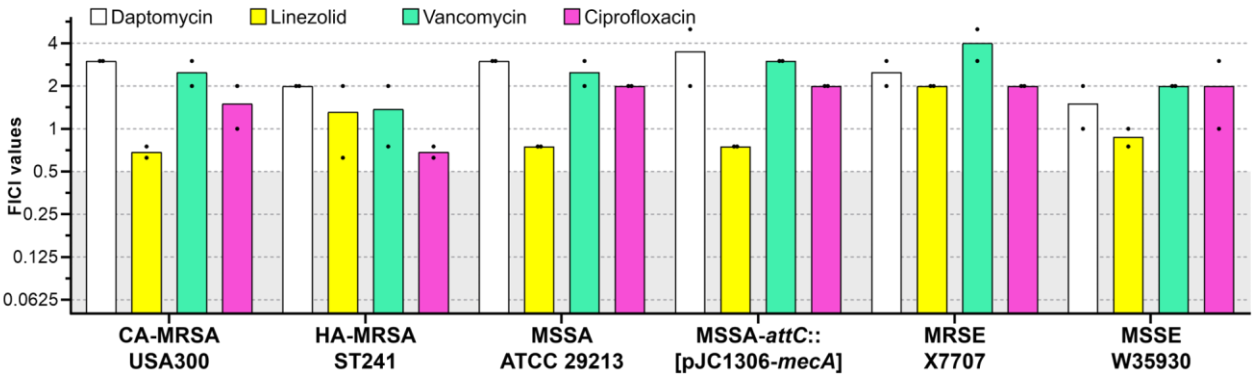

Figure S2. cPAC fails to potentiate non-β-lactam antibiotics against *Staphylococci* strains. Checkerboard assays were done for the combination of cPAC with daptomycin (lipopeptide), linezolid (oxazolidinone), vancomycin (glycopeptide) and ciprofloxacin (fluoroquinolone) to determine the FICI for each combination in both *S. aureus* and *S. epidermidis* clinical strains. Bars represent the average of two independent replicates.

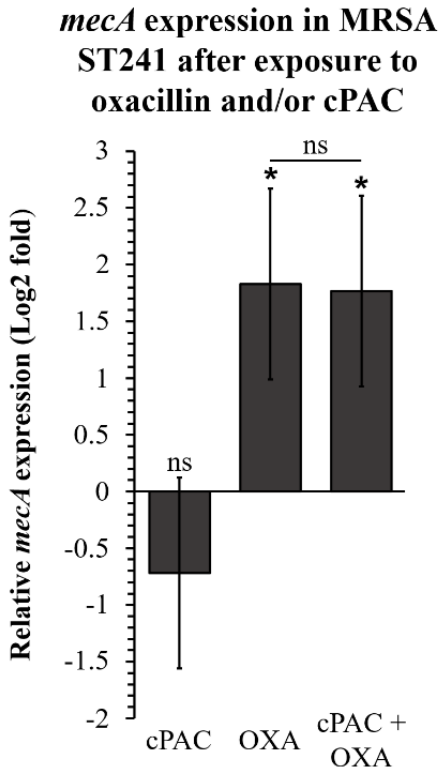

Figure S3. Relative expression of *mecA* gene by RT-PCR in MRSA ST241 strain. The mRNA levels shown are relative to those obtained without treatment. ***** $P < 0.05$, not significant, 4 independent biological replicates, 2 technical replicates. Each condition was compared to control (no treatment) and the error bars show the SD. OXA: oxacillin 1 µg/mL, cPAC: cPAC 25 µg/mL.