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Carvacrol Enhances the Antimicrobial Potency of Berberine in *Bacillus subtilis*

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Abstract

The essential oil carvacrol from oregano displays a wide range of biological activities among which is found the inhibition of efflux pumps. Thus, using carvacrol, the current work undertook the effort to potentiate the antimicrobial activity of berberine, a natural product with limited antimicrobial efficacy due to its efflux. Following the selection of concentrations for the combinatorial treatments, guided by checkerboard microtiter plate assay and growth experiments, ethidium bromide accumulation assay was used to find that 25 μg mL^{-1} carvacrol displayed a weak efflux pump inhibitor character in *Bacillus subtilis*. Scanning electron microscopy images and cellular material leakage assays showed that carvacrol at this concentration neither altered the morphology nor the permeability of the membrane alone but when combined with 75 μg mL^{-1} berberine. Among the efflux pumps of different families found in *B. subtilis*, except for BmrA and Mdr, the increase in the expressional changes was striking, with Blt displaying ~ 4500-fold increase in expression under the combination treatment. Overall, the findings demonstrated that carvacrol potentiated the effect of berberine; however, not only multiple pumps but also different targets may be responsible for the observed activity.

Introduction

Misuse and overuse of available drugs have led to a worldwide crisis in antimicrobial resistance. Evolving resistance mechanisms dangerously increase morbidity, mortality, and related economic burden [1]. Today, antimicrobial resistance is one of the major challenges faced in both clinical settings and societies [2]. Resistance against multiple drugs may also be developed, making the situation more challenging to cope with [3]. Such resistance mechanisms are either developed naturally or acquired subsequently. Natural or intrinsic mechanisms include altering the target to show low or no affinity, lowering cell permeability, inactivating antibiotics, and mediating active drug efflux. Mutations and resistance gene transfers are known as acquired mechanisms [4]. Among different mechanisms, drug efflux, which prevents intracellular drug accumulation, stands out as one of the most prominent strategies [5]. The expression of such efflux systems is commonly induced in response to the presence of antimicrobials [6].

The decline in the potency of readily available drugs and the decrease in the new antimicrobial discovery rate necessitate the introduction of new molecules or novel approaches in clinical use to fight against antimicrobial

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resistance [7]. Plant-derived chemicals are intriguing molecules in this fight; nevertheless, they have received far less attention in this respect. With the development of techniques to elucidate their targets and action mechanisms, they are now regaining their attention as sources of therapeutical agents. Among these molecules is the plant alkaloid berberine, which exhibits antimicrobial activity [8]. Owing to its lower cytotoxicity and lower mutagenicity toward human cells, it has been attractive in clinical settings. However, its extrusion through efflux pumps significantly lowers its activity and restricts its potential [9].

In this context, one effective strategy is the combinatorial therapies of bioactive molecules of low efficacy with efflux pump inhibitors (EPIs). Carvacrol is among the plant essential oils which are attractive in enhancing the activities of antibiotics [10]. Carvacrol is primarily used in food and cosmetic industries [11]; but due to the wide range of biological activities (antimicrobial, antioxidant, anticancer, etc.) it displays, it has attracted attention for clinical studies [12]. Its principal target is the bacterial membrane but it also has other targets which are commonly neglected [13]. Interestingly, Miladi et al. (2016) ascribed an EPI role to carvacrol in food-borne pathogens after demonstrating that it inhibited ethidium bromide (EtBr) efflux [14]. Very recently, Barbosa et al. (2021) showed that carvacrol enhances the activity of norfloxacin in *Staphylococcus aureus*, possibly by targeting the NorA efflux pump [15].

The relative safety of carvacrol [16] has motivated the work to evaluate carvacrol as an EPI for the different classes of efflux pumps in the Gram-positive model organism, *Bacillus subtilis*, to potentiate the antibacterial activity of berberine. Different approaches were then undertaken to establish the mechanism of carvacrol in the combinatorial treatment.

Materials and Methods

Bacterial Strains and Chemicals

Wild-type *B. subtilis* 168 (DSM 402) was used to evaluate the EPI property of carvacrol. Carvacrol (CAS No. 499–75-2) and berberine chloride hydrate (CAS No. 141433–60-5) were purchased from Sigma-Aldrich. Carvacrol stock solution (800 μg mL^{-1}) was prepared in dimethyl sulfoxide (DMSO), and berberine stock solution (1200 μg mL^{-1}) was prepared in 50% DMSO. Dilution was achieved in nutrient broth (peptone from meat 5 g/L; beef extract 3 g/L) (NB, Merck). All other chemicals, reagents, and kits used were obtained from Bio-Rad (Hercules, CA), Merck, Qiagen (Valencia, CA), Fermentas, or Roche.

Growth Conditions for Carvacrol and Berberine Treatment

Cells were grown in NB at 37 °C and 180 rpm and treated with berberine and carvacrol (alone or in combination at different concentrations) as OD_{600} reached 0.45–0.55. Control cells were treated with DMSO at a final concentration of 0.6% (v/v), which was identical to the DMSO concentration in carvacrol/berberine-treated cultures. Growth was monitored spectrophotometrically by measuring the OD_{600} every hour. The presented results are the means of triplicate experiments given with error bars.

Determination of Minimum Inhibitory Concentration and Combinatorial Effect

Minimum inhibitory concentration (MIC) of the berberine–carvacrol combination was assessed using checkerboard microtiter plate assay [17, 18]. Two fold serial dilutions of berberine from 300 μg mL^{-1} to 4.69 μg mL^{-1} and carvacrol from 200 μg mL^{-1} to 3.125 μg mL^{-1} were prepared with NB in sterile 96-well U-bottom plates. A single column of the test plate was prepared with serial dilutions of the solvent DMSO as control, with the highest concentration of 50% (v/v) in the first well. Each well was inoculated with 1×10^5 CFU mL^{-1} cells, and the plates were incubated at 37 °C for 24 h. The assay was performed in duplicate.

After a 24-h incubation, 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, Germany) dye reduction test was used to select the wells with no visible cell growth. TTC solution was added to each well at a final concentration of 0.5% (w/v). Plates were incubated for 1 h at 37 °C. To determine the MIC, wells were assessed based on the color change. The development of red color indicated the presence of viable cells in the wells.

Scanning Electron Microscopy

Samples for scanning electron microscopy (SEM) were prepared based on a previously reported protocol [19]. Cells were treated with 75 μg mL^{-1} berberine, 25 μg mL^{-1} carvacrol, or the combination of these for 3 h. SEM images were obtained by Philips XL30 ESEMFEG/ EDAX system (Philips, Holland) under high vacuum mode with 10 kV energy and 3.0 spot size. Cell lengths were calculated by averaging the sizes of at least eight cells from each image.

Release of Cytoplasmic Material

Cytoplasmic material release was monitored spectrophotometrically [20]. Overnight grown cells were transferred

into 200 mL fresh NB at a ratio of 1:100. Cells were grown at 37 °C and 180 rpm until OD_{600} reached 0.5 and centrifuged at 3,000 *g* for 15 min. Supernatant was discarded, and the pellet was washed three times with phosphate-buffered saline (PBS) and then resuspended in 1 mL PBS. 4 mL PBS containing 25 or 200 μg mL^{-1} carvacrol was prepared in 25 mL Erlenmeyer flasks. 200 μL of the cell suspension with approximately 4×10^8 CFU mL^{-1} of cells was used to inoculate carvacrol-containing PBS in the two flasks. PBS with an equal volume of DMSO (2% v/v) but without carvacrol was used as the control. All cell suspensions were incubated at 37 °C and 180 rpm. 1 mL of sample from each flask was taken every 30 min and centrifuged at 6,000 *g* for 2 min to precipitate the cell. Cytoplasmic material in the supernatants was detected by measuring absorbance at 260 nm. The assay was performed in triplicate and the results are presented as means with error bars.

Ethidium Bromide Accumulation Assay

EtBr accumulation was measured on 96-well plates using a Synergy HTX Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT, USA) equipped with filters of 540 and 590 nm for excitation and emission, respectively. Fluorescent intensities were evaluated relatively in berberine–carvacrol treated and control cells using a modified version of the previously reported method [21, 22]. Overnight grown cells were inoculated into 5 mL fresh NB. Cell growth was achieved at 37 °C and 180 rpm. As OD_{600} reached 0.5, cells were centrifuged at 2,000 *g* and 4 °C for 4 min. Cell pellets were suspended in 2 mL of 0.35 M NaCl. 180 μL of the cell suspension was mixed with 50 mM KP_i, 5 mM $MgSO_4$, and 25 mM glucose. Immediately after the addition of glucose, 10 μM of EtBr (Invitrogen, California, USA) was added to the mixture and fluorescence was recorded for 20 min. To measure EPI activity, 25 μg mL^{-1} carvacrol was added to the cell suspension before glucose. All wells contained 0.5% (v/v) DMSO. The assay was performed in triplicate.

Gene Expression Analysis by Quantitative Polymerase Chain Reaction

Total RNA was isolated from 1-h DMSO, carvacrol, berberine, and berberine–carvacrol-treated cells for the analysis of gene expression since longer periods of treatment adversely affected cell viability and reduced RNA quality. Collected samples were mixed with RNAprotect Bacteria Reagent (Qiagen, Germany) and incubated at room temperature for 5 min. Cell suspensions were then centrifuged at 5,000 *g* for 10 min. Cell pellets were subjected to mechanical disruption at 6 m/s for 60 s using FastPrep-24 (MP Biomedicals, USA) instrument and then RNeasy Mini Kit (Qiagen, Germany) was used for total RNA isolation.

Primers for quantitative polymerase chain reaction (qPCR) were designed with Primer Premier 6.0 software and are given in the supplemental file (Table S1). 16S ribosomal RNA, *rrnA*-16S, was selected as the housekeeping gene. cDNAs were synthesized using QuantiTech Reverse Transcription Kit (Qiagen, Germany). Reactions were carried out in a total volume of 20 μL with 5 ng cDNA, 4 μL of 5X SYBR Green I mix (LightCycler FastStart DNA MasterPLUS SYBR Green I, Roche, Germany), and 100 nM of primers using LightCycler 1.5 Instrument (Roche, Germany). The following parameters were used for amplification: 95 °C for 10 min, followed by 40 cycles of denaturation for 10 s at 95 °C, annealing for 10 s at T_a (Table S1), and extension for 45 s at 72 °C. All reactions were performed in triplicate and the obtained Ct values are averaged to calculate fold changes based on the $2^{-\Delta\Delta CT}$ formula [23].

Results

Combinatorial Effect of Berberine and Carvacrol on Cell Growth

The combinatorial effect of carvacrol and berberine was first evaluated with a checkerboard microtiter plate assay. A test plate example is given in the supplemental file (Fig. S1).

MIC for carvacrol was found as 200 μg mL^{-1}. For berberine, MIC lies between 150 and 300 μg mL^{-1}, being closer to 200 μg mL^{-1}. These values are consistent with literature findings [24–26]. In the berberine–carvacrol combinations of 37.5 and 100 μg mL^{-1} and 150 and 3.125 μg mL^{-1}, there was no growth. These values were used to design further experiments. In order to circumvent or minimize the effect of carvacrol on the membrane and emphasize its EPI feature, its concentration should be finely tuned. Guided by the checkerboard microtiter plate assay, 200 μg mL^{-1} was taken as the upper limit and the effect of carvacrol on cell growth was investigated (Fig. 1a). Up to 100 μg mL^{-1}, there was no inhibitory effect on cell growth. As carvacrol concentration was raised to 150 μg mL^{-1}, growth was significantly retarded. Consistent with the checkerboard assay, in the presence of 200 μg mL^{-1} carvacrol, growth completely ceased. This should be the concentration at which it permeabilizes and disrupts the membrane. The obtained growth profiles have shown that ≤100 μg mL^{-1} carvacrol is suitable in assays with berberine for testing its EPI feature since it did not alter growth.

Taking this value as the threshold concentration for carvacrol, growth experiments with the berberine–carvacrol combination were designed. In our previous work, 75 μg mL^{-1} berberine has been shown to only slightly affect cell growth [19]; therefore, varying carvacrol concentrations were combined with 75 μg mL^{-1} berberine. In order

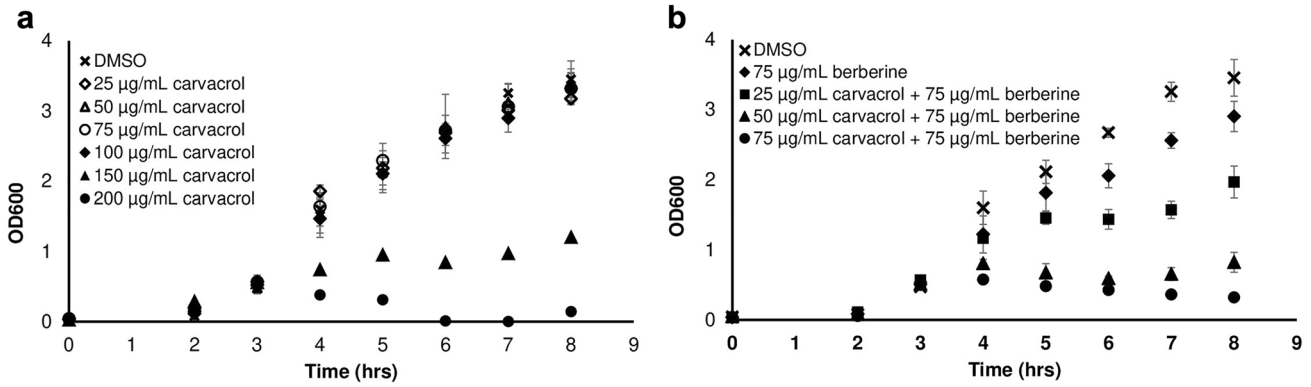

Fig. 1 Growth of *B. subtilis* in the presence of increasing concentrations of carvacrol (C) (**a**), in the presence of berberine (B), and increasing concentrations of carvacrol (**b**) (DMSO concentration was set at 0.6% (v/v) for both control and berberine and/or carvacrol-treated cells.)

to bring out the EPI feature of carvacrol, its concentration was not allowed to exceed the concentration of berberine; therefore, the maximum concentration of carvacrol was set at 75 µg mL^{-1}.

Figure 1b displays growth profiles of cells treated with different berberine–carvacrol combinations. The results were pretty consistent with the checkerboard assay. Growth was moderately affected with the 75 µg mL^{-1} berberine and 25 µg mL^{-1} carvacrol combination while increasing the carvacrol concentration to 50 µg mL^{-1} and above severely retarded growth. The availability of viable cells is important in the characterization of treated cells; therefore, 25 µg mL^{-1} carvacrol was selected as the final concentration to be combined with berberine in further experiments. The following assays were conducted to understand the mechanism of the combination treatment.

Imaging Bacterial Morphology

SEM images were taken to get descriptive information about the morphological changes in the carvacrol and/or berberine-treated cells. Three hours after treatment, growth profiles of treated cells were significantly different from the control cells (Fig. 2); therefore, images were taken from 3-h treated cells.

Images have shown that control cells had pretty much smooth surfaces (Fig. 2a). The average cell length was 4.50 ± 0.54 µm. Upon carvacrol treatment (Fig. 2b), few dimples developed on cell surfaces, and cells were slightly crumpled when compared to control cells. Furthermore, they were somewhat shorter (3.93 ± 0.72 µm). Surfaces of berberine-treated cells (Fig. 2c) were largely crumpled compared to control cells and there were also blebs and dimples, as previously described [19]. There were similar changes on the surfaces of cells treated with the berberine–carvacrol combination (Fig. 2d). Berberine-treated cells, as well as berberine–carvacrol-treated cells were both longer than

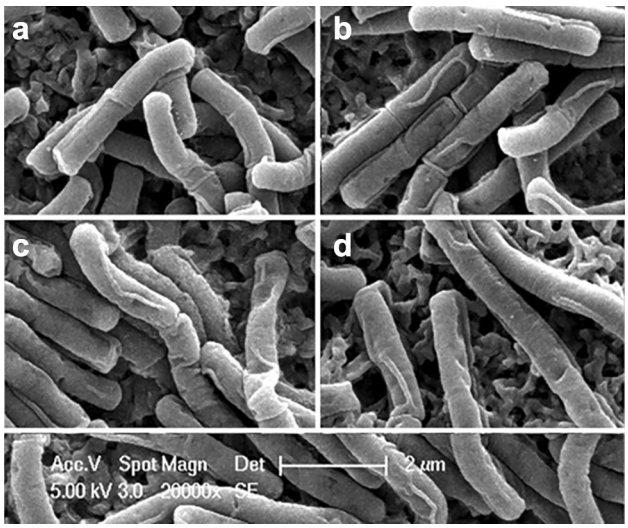

Fig. 2 SEM images of control (**a**), carvacrol-treated (**b**), berberine-treated (**c**), and berberine and carvacrol combination-treated (**d**) cells

control cells (5.75 ± 0.86 µm and 5.69 ± 0.80 µm, respectively). The inhibition of the cell division protein, FtsZ, in the presence of berberine is a possible explanation for the observed elongated sizes [27].

Analysis of Membrane Permeability

The hydrophobic character of carvacrol enables it to penetrate the lipid membranes and damage their integrity, resulting in the formation of pores and concomitant release of intracellular materials to the extracellular environment [26]. Therefore, the possibility of the selected carvacrol concentration to promote the breakdown in permeability function was next investigated. This activity was monitored via spectrophotometric measurement of the UV-absorbing material under carvacrol treatment (Fig. 3).

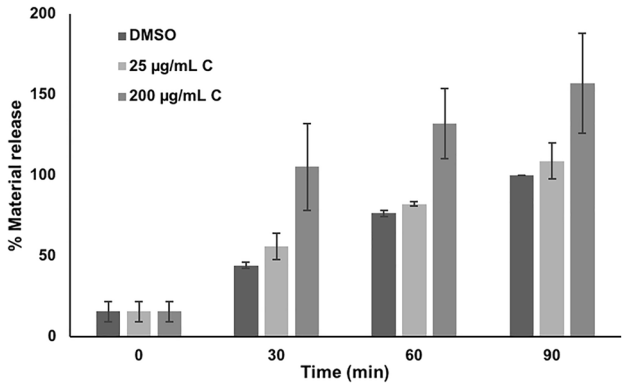

Fig. 3 Measurement of intracellular material release with carvacrol (C). Cellular material release in cells treated with DMSO (control) 90 min after treatment was taken as 100%

In the presence of 25 µg mL^{-1} carvacrol, leakage of the cellular material was almost negligible when compared to control cells. However, when cells were treated with 200 µg mL^{-1} carvacrol, cellular material leakage was extremely high and fast, a serious indication of damaged cell membrane [28]. While 25 µg mL^{-1} carvacrol caused no major damage to the membrane, 200 µg mL^{-1} carvacrol completely distorted the membrane structure.

Effect on Efflux Pumps

SEM images and measurements of cellular material leakage demonstrated that there was no significant change in the cell membrane with 25 µg mL^{-1} carvacrol. Consequently, the EPI potential of carvacrol was investigated using the EtBr accumulation technique at this concentration.

Figure 4 shows that the recorded fluorescence intensity due to EtBr accumulation was slightly higher in the presence of 25 µg mL^{-1} carvacrol. This suggested that carvacrol inhibited EtBr efflux, though moderately. The fact that carvacrol leads to membrane depolarization [29, 30] may also be correlated with this efflux pump inhibition.

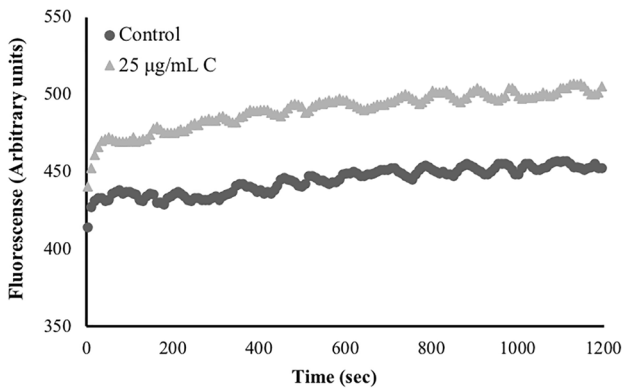

Fig. 4 Fluorometric measurements of EtBr accumulation in *B. subtilis* cells in the presence of 25 µg mL^{-1} carvacrol (C)

To understand this observed behavior, the fold changes in major efflux pumps were measured. The results obtained are presented in Table 1. When the cells were treated with berberine or carvacrol alone, the expression of all the selected pumps increased moderately. Overall, the expressional changes were slightly more significant under berberine treatment. The fact that berberine is an efflux pump substrate could have triggered this. On the other hand, when the cells were treated with the combination, apart from BmrA and Mdr, the increase in the expressional changes of pumps was striking, with Blt displaying ~ 4500-fold increase.

Discussion

Until today, different synthetic and natural compounds have been explored as EPIs for combinatorial therapies to enhance the biological activities of different antimicrobials [31]. One famous synthetic EPI, phenylalanine-arginine β-naphthylamide, is often used in combination with fluoroquinolone antibiotics to treat clinically challenging *Pseudomonas aeruginosa* infections [32]. A number of plant-derived bioactive molecules, such as reserpine, curcumin, and piperine, have been evaluated for their EPI properties [33]. Among these, the alkaloid reserpine inhibits the eukaryotic efflux pump P-glycoprotein and its homologs in bacteria, such as BmrA of *B. subtilis* [22, 34]. The flavonolignan silibinin has been reported to inhibit NorA of *S. aureus* [35]. Unfortunately, the cytotoxicity displayed by these natural products is their major drawback; thus, search for new EPI candidates continues. To this end, the safety profile of carvacrol [16, 36] has encouraged us to use it as an EPI to enhance the antimicrobial activity of berberine. Although berberine has long been a part of traditional medicine for the treatment of inflammatory disorders, skin diseases, wound healing, reducing fevers, affections of eyes, treatment of tumors, digestive and respiratory diseases, and microbial pathologies [37], it is not yet a part of systematic treatment.

Table 1 Expressional changes of selected efflux pump genes

Efflux pump		Fold change ($2^{-\Delta\Delta CT}$)		
Gene name	Family	Carvacrol	Berberine	Carvacrol&Berberine
blt	MFS	3.3	11.7	4449.7
bmr	MFS	9.6	6.2	112.0
lmrB	MFS	4.6	19.0	250.1
mdr	MFS	4.9	20.8	19.0
bmrA	ABC	2.2	1.7	7.2
ebrA	SMR	3.4	18.4	64.3
ebrB	SMR	9.9	11.9	353.7

Many studies investigating the EPI properties of carvacrol commonly only monitor EtBr [14] or Nile red [38] accumulation as a proof of concept. While EtBr binds intracellular DNA and causes fluorescence, Nile red is a lipophilic dye that stains intracellular lipids and becomes strongly fluorescent. Other studies use combinatorial treatments with well-known antimicrobials such as tetracycline, norfloxacin [14, 39], benzalkonium chloride [14], or nalidixic acid [40] to show that carvacrol indeed lowers the MIC of these known drugs. Here, we have selected berberine as a substrate for the combination treatment due to its low efficacy, but non-negligible antimicrobial potential, and have shown that carvacrol can enhance its antimicrobial activity. Increasing the efficacy of berberine with a clinically safe EPI could increase its potential for clinical settings.

Studies on the mechanistic details of the antimicrobial activity of carvacrol are available [26, 41–45]; however, among these only Chueca et al. [44] and Pesingi et al. [45] have reported its relation to efflux pumps to a certain extent. Chueca et al. [44] have analyzed the transcriptional response of *Escherichia coli* MG1655 under carvacrol treatment and reported a relation with genes encoding AcrAB-TolC multidrug efflux system in aiding the cells to cope with carvacrol stress. Similarly, Pesingi et al. [45] reported the role of MexAB-OprM efflux pump of *P. aeruginosa* in carvacrol resistance when considered as an antimicrobial agent. Studies focusing on its EPI potential would not go much beyond the calculation of MIC values [14, 39, 46]. Only very recently Barbosa et al. [15] provided direct evidence for the inhibition of NorA of *S. aureus* by carvacrol. They suggested that carvacrol competes with the substrates of the pump and keeps the pump busy by being continuously excreted from the bacterial cells in place of the antibiotic. Thus, this clues that MexAB-OprM may act in the same way since carvacrol is its substrate [45].

The approach employed here was first to characterize the conditions where carvacrol can be considered to be an EPI with berberine in *B. subtilis* and then to measure expressional changes in the different efflux pumps under the selected condition. Among carvacrol targets in other bacteria, while NorA of *S. aureus* belongs to the major facilitator superfamily (MFS), AcrAB-TolC of *E. coli* and MexAB-OprM of *P. aeruginosa* belong to the resistance nodulation division (RND) family of efflux pumps. Since it is clear that carvacrol may target different efflux pumps, well-known pumps of different families as Blt, Bmr, Mdr, and LmrB of the MFS-type, EbrAB of the SMR type, and BmrA of the ABC-type [22, 47] have been selected for analysis in *B. subtilis*. Unlike the tetracycline efflux transporters, these selected pumps are all less selective, associated with the efflux of not only a single substrate but of a variety of toxic substances. Although most pumps displayed increased expression upon combination treatment, this is not unexpected since most EPIs target multiple pumps [48], all having substantial roles in efflux. However, the increased expression in *blt* is worth emphasizing. Just like NorA, Blt belongs to the MFS family of pumps. Under standard conditions, the transcription of *blt* has been reported to be undetectable [49]; however, its increased expression has been reported to lead to a multidrug-resistant phenotype [50].

Conclusion

The results demonstrated that carvacrol has the potential to be used in combination therapies with antimicrobials, such as berberine, displaying low efficacy due to the action of efflux pumps. The data obtained cannot directly identify the pump(s) targeted by carvacrol; nevertheless, they indicate that Blt should have a significant role. While it may inhibit Blt, it may as well compete with other substrates for Blt and keep it busy. Our findings may pave the way for further studies on the use of carvacrol with other Blt substrates for antimicrobial purposes. Given that *B. subtilis* is a model organism, it may also be advantageous to examine these findings in other organisms harboring Blt homologs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-022-02823-7.

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Author Contributions BSA, FGA, and NAS conceptualized and designed the study. BA and CSA performed the experiments. BA and EO analyzed the data. BSA, BA, and KU wrote the first draft. All authors critically revised the manuscript and approved the final submitted version.

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Data Availability All data are available in the text.

Code Availability Not applicable.

Declarations

Conflicts of interest The authors declare no competing interests.

Ethics Approval Not applicable.

Informed Consent Not applicable.

Consent for Publication All authors agree on the submission of the manuscript.

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