



Article

Comparison of Antibacterial Activity of Phytochemicals against Common Foodborne Pathogens and Potential for Selection of Resistance

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Abstract: Antimicrobial resistance is now commonly observed in bacterial isolates from multiple settings, compromising the efficacy of current antimicrobial agents. Therefore, there is an urgent requirement for efficacious novel antimicrobials to be used as therapeutics, prophylactically or as preservatives. One promising source of novel antimicrobial chemicals is phytochemicals, which are secondary metabolites produced by plants for numerous purposes, including antimicrobial defence. In this report, we compare the bioactivity of a range of phytochemical compounds, testing their ability to directly inhibit growth or to potentiate other antimicrobials against *Salmonella enterica* Typhimurium, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Staphylococcus aureus*. We found that nine compounds displayed consistent bioactivity either as direct antimicrobials or as potentiators. Thymol at 0.5 mg/mL showed the greatest antimicrobial effect and significantly reduced the growth of all species, reducing viable cell populations by 66.8%, 43.2%, 29.5%, and 70.2% against *S. enterica* Typhimurium, *S. aureus*, *P. aeruginosa*, and *L. monocytogenes*, respectively. Selection of mutants with decreased susceptibility to thymol was possible for three of the pathogens, at a calculated rate of 3.77×10^{-8} , and characterisation of *S. enterica* Typhimurium mutants showed a low-level MDR phenotype due to over-expression of the major efflux system AcrAB-TolC. These data show that phytochemicals can have strong antimicrobial activity, but emergence of resistance should be evaluated in any further development.

Keywords: antimicrobial resistance; AMR; phenolics; flavonoids; natural products



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1. Introduction

Bacterial infections remain a major cause of illness and mortality for humans and animals, including those caused by foodborne pathogens ingested through contaminated foodstuffs and feedstocks [1]. This impact is being worsened by the increasing numbers of pathogenic microorganisms demonstrating antimicrobial resistance (AMR) against antibiotics used for clinical and industrial purposes [2–4]. In parallel with this increase in AMR, there has been a decline in the development of new antibiotics, making novel antimicrobials urgently required [5].

Plants, a historically productive source of natural pharmacological compounds, represent a source of novel biochemical structures [5], and numerous plant extracts have shown direct in vitro antimicrobial activity [6–11]. Much of this bioactivity is the result of phytochemicals, secondary metabolite compounds produced by plants for multiple functions, including antimicrobial defence against microbial pathogens [12–15]. With over 8000 compounds identified [14], phytochemicals display enormous structural diversity [15,16]. As well as the identification of direct antimicrobial activity, including carvacrol and cinnamic

acid against *S. Typhimurium* and *Escherichia coli* [17] and the coumarin cajanuslactone against *S. aureus* [18], phytochemicals have also been identified that synergise with other antimicrobials via direct synergism [19,20] and the capacity to potentiate their activity via the inhibition of efflux pumps, etc. [21–24].

Despite this body of evidence demonstrating the antimicrobial properties and potential of phytochemicals, few studies have focused on responses of microorganisms to phytochemical challenge [25–29]. If any phytochemicals are to be developed and applied practically, a good understanding of the responses of target organisms is needed. In this study, we determined the direct inhibitive and synergistic activities of a panel of phytochemicals against standard laboratory reference strains of the common foodborne pathogens *S. Typhimurium*, *S. aureus*, *P. aeruginosa*, and *L. monocytogenes*. We then assessed the potential of the most potent compounds to select for resistant mutants, which were then characterised phenotypically and genotypically.

2. Materials and Methods

2.1. Bacterial Strains

The following bacterial strains were used: *Salmonella enterica* serovar Typhimurium 14028S, *Staphylococcus aureus* NCTC 8532, *Pseudomonas aeruginosa* PA14, and *Listeria monocytogenes* LM014. All strains were stored in Protect Microorganism Preservation System ceramic cryo-beads (Technical Service Consultants Ltd., Heywood, UK) at $-70\text{ }^{\circ}\text{C}$ until required. One cryo-bead from a stock was used to inoculate 5 mL of sterile growth medium when overnight cultures were needed; *S. Typhimurium*, *S. aureus*, and *P. aeruginosa* strains were cultured at $37\text{ }^{\circ}\text{C}$ in Luria-Bertani Broth (LB) (Fisher Scientific BioReagents, Loughborough, UK), whereas *L. monocytogenes* was cultured at $37\text{ }^{\circ}\text{C}$ in Brain–Heart Infusion (BHI) Broth (Oxoid, Basingstoke, UK).

2.2. Phytochemicals

A panel of phytochemicals consisting of caffeic acid, cinnamic acid, ferulic acid, hesperidin, kaempferol, naringenin, naringin, quercetin, rutin, thymol, vanillic acid, and vanillin was sourced from Sigma-Aldrich (Gillingham, UK) and eriodictyol from Extrasynthase (Genay, France). All compounds were stored as suggested by the manufacturer. Prosur NATPRE T-10+, chosen because it is a commercially available phytochemical mixture designed to substitute sodium nitrite/nitrate as a food preservative, was stored in aluminium foil-wrapped Durans, sealed with parafilm, and kept in a dark environment. All phytochemicals were dissolved at room temperature in DMSO, vortexed, and stored at $4\text{ }^{\circ}\text{C}$. Compounds were selected based on the rationale of balancing previously described activity and the general cost of synthesis/purchase. Compounds representative of most phytochemical structural families were selected. Tested phytochemical concentrations were selected to reflect the working concentrations of the commercially available Prosur NATPRE T-10+ mixture and not those found in natural extracts.

2.3. Growth Inhibition Assays

A high-throughput screen was used to test the panel of all phytochemicals for antibacterial activity. From 1 mg/mL phytochemical working stocks, dilutions to give final concentrations of 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL were made and 100 μL of each were added to three wells of 96-well microtiter plates. Plates were then inoculated with bacteria diluted in 100 μL of medium (LB for *S. Typhimurium*, *S. aureus*, *P. aeruginosa* strains; BHI for *L. monocytogenes*) at $\sim 10^{-5}$ CFU/mL. Drug-free media controls were included as well as broth containing DMSO at the final concentrations used as solvent controls. Plates were sealed with gas-permeable membranes (ThermoFisher Scientific, Cambridge, UK) and incubated at $37\text{ }^{\circ}\text{C}$ overnight for 16 h. After incubation, the $\text{OD}_{(600\text{ nm})}$ of each culture was measured using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Endpoint $\text{OD}_{(600\text{ nm})}$ readings were used instead of a time-course experiment to allow for a

more rapid screening of multiple plates at once. All experiments were replicated four times and statistically analysed as detailed below (see Section 2.10).

2.4. Identification of Antimicrobial Potentiation

The ability of each phytochemical to synergise with chloramphenicol (chosen as an antibiotic whose activity is increased by agents that enhance membrane permeability or inhibit efflux) was determined. Plates were inoculated with phytochemicals as described in Section 2.3 above, but the media used to inoculate bacteria contained chloramphenicol at a final concentration of 2 µg/mL. Plates were incubated and analysed as for direct growth inhibition assays; phenyl-arginine β-naphthylamide (PAβN) was included as a control, being a known efflux pump inhibitor. All experiments were replicated four times and analysed as detailed below. Phytochemicals that displayed significant reduction in OD_(600 nm) measurements with the inclusion of chloramphenicol, but not in its absence (at least not to the same extent), were deemed as holding the capacity for antibiotic potentiation.

2.5. Measuring Cell Viability after Phytochemical Exposure

To distinguish bacteriostatic and bactericidal activity, the viable numbers of cells after exposure to a panel of selected phytochemicals (chosen based on results from the high throughput end-point assay above) were determined. Overnight bacterial cultures were set up in triplicate for each species. The next day, fresh growth media was supplemented with phytochemicals at either 0.05 mg/mL or 0.5 mg/mL and inoculated with ~10⁵ CFU/mL of each species (overnight bacterial cultures were diluted appropriately into 250 µL of PBS). These cultures were then sampled by removing 20 µL at 0, 1, 2, 4, 8, and 16 h of incubation at 37 °C and ~200 rpm. Each aliquot was serially diluted and 20 µL spots inoculated onto LB agar plates and incubated overnight at 37 °C before colony-forming units (CFU) were enumerated. In total, three technical replicates for each of three biological replicates were tested in this way and analysed as detailed below.

2.6. Drug Accumulation Assays

To determine whether any phytochemicals impacted cellular permeability, the accumulation of resazurin, a molecule that is metabolised within the cell into a fluorescent product, was measured. Intracellular accumulation of resazurin is usually limited due to low permeability, and it is also a substrate for efflux pumps. Accumulation was measured in the presence and absence of the selected phytochemicals. Overnight bacterial cultures were grown, then used to incubate fresh growth media the following day. Once bacterial cultures had reached an OD_(600 nm) of 0.2–0.5, cells were harvested and resuspended in sterile PBS, normalising the OD_(600 nm) to the lowest measurement taken. A 96-well plate was inoculated with 5 µL of a 400 µg/mL resazurin stock (20 µL of a 100 µM ethidium bromide stock was substituted for *S. aureus* and *L. monocytogenes*), as well as 2 µL of sterile PBS/DMSO/50 mg/mL phytochemical stock depending on the experimental condition. Positive controls included an additional 5 µL of a 5 mg/mL PAβN stock and, finally, 193 µL of bacterial suspensions were added to the appropriate wells and mixed. In total, five technical replicates for each of three biological replicates were tested in this way. As soon as all wells were filled, a transparent membrane was sealed onto the 96-well plate, which was placed into a FLUOstar Omega Plate Reader to measure OD_(600 nm) and fluorescence readings (excitation: 544 nm, emission: 590 nm) over a period of 16 h.

2.7. Mutant Selection

Phytochemical-laced agar was inoculated with either *S. Typhimurium*, *S. aureus*, *P. aeruginosa*, or *L. monocytogenes* and incubated to select for resistant mutants. Muller Hinton agar plates were prepared and supplemented with either caffeic acid, thymol, or the Prosur NATPRE T-10+ mix at 1× and 2× the MIC. These phytochemicals were selected due to their potent directly antimicrobial activity, their capacity for the potentiation of chloramphenicol, or their current use as a food preservative substitute. Supplemented

plates were then inoculated with bacteria concentrated to 10^{-10} or 10^{-9} CFU in 100 μ L volumes and incubated at 37 °C overnight. A dense inoculum was used as the classic methodology to encourage and capture the potential for mutant selection from exposure to the tested compounds/mixture. Control plates with no phytochemicals or with DMSO as a vehicle control were prepared in parallel. The next day, the plates were enumerated, the average mutation frequency was calculated (see below), and random mutant colonies were picked via toothpick before culturing and storage in 40% glycerol stocks at -70 °C. Plates that showed no colony growth were incubated for a further 24 h, then re-examined.

2.8. Mutant Sequencing and Phenotyping

Mutant strains of *S. Typhimurium* selected by phytochemical exposure were cultured for DNA extraction and sequencing as described recently [30]. Briefly, cultured cells were lysed and DNA was isolated via DNA-binding magnetic beads (KAPA Pure Beads, Roche Diagnostics) and eluted with 10 mM Tris-Cl, pH 8.5. The extracted DNA samples were sequenced using an Illumina Nextseq500 instrument [30] and sequence reads were quality filtered using Trimmomatic (v3.5) with default parameters. Contigs were assembled using SPAdes v3.11.1 and the de novo and parental genomes fed through the Snippy v3.1 software to identify single nucleotide polymorphisms (SNPs) [30]. The sequenced mutant strains were also phenotypically assayed using the growth analysis and drug accumulation assays described above, and the MICs of antibiotics kanamycin, tetracycline, ampicillin, chloramphenicol, and nalidixic acid were also determined via the microdilution broth method. Finally, to identify whether mutant strains had any difference in biofilm-forming ability, crystal violet assays and analysis of colony morphology on congo red plates were used, both as described previously [31].

2.9. Transmission Electron Microscopy (TEM)

An overnight culture of *S. Typhimurium* and a previously selected thymol-tolerant mutant strain were used to inoculate 200 mL LB within Erlenmeyer flasks in a 1:200 ratio. After an overnight incubation at 37 °C and \sim 200 rpm, these flask cultures were separated into 30 mL aliquots within 50 mL centrifuge tubes and supplemented to contain 0.25–1 mg/mL thymol, with PBS and DMSO controls included. These samples were incubated at room temperature for two hours before being pelleted, washed in 10 mL PBS, and pelleted again to be covered with 1 mL PBS. The pelleted samples were then processed by the QIB Bioimaging Core Facility for sample fixing and TEM imaging. Briefly, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (both Agar Scientific Ltd., Stansted, UK) was used as the fixative, post fixed in 1% osmium tetroxide (Agar Scientific Ltd., UK) for two hours, and dehydrated through an ethanol series (30–90% for 15 min each, followed by 100% ethanol three times each for 15 min). Finally, samples were embedded in resin through a series mix of LR White medium-grade resin (Agar Scientific Ltd., Stansted UK) and 100% ethanol (1:1, 2:1, 3:1 resin:ethanol, and, finally, three times 100% resin, each for at least 1 h with the final 100% resin step overnight), before polymerising overnight at 60 °C and sectioning the samples to \sim 90 nm thickness using an ultramicrotome (Leica EM UC6, Wetzlar, Germany) onto carbon-coated copper TEM grids (EM Resolutions Ltd., Keele, UK) and sequential staining with \sim 2% uranyl acetate (BDH 10288) and 0.5% lead citrate-tribasic trihydrate (Sigma 153265-25G). Sections were examined and imaged in an FEITalos F200C transmission electron microscope at 200 kV with a Gatan One View digital camera. Digital micrograph files (.DM4) were converted to a .TIFF format for ease of viewing. The resulting images were parsed to identify representative examples of the bacterial morphologies and analysed as described below.

2.10. Statistical Analyses

For all of the previously described experiments, the following statistical analyses were implemented. For the growth inhibition assays and identification of antimicrobial potentiation, all experiments were statistically analysed via a one-way ANOVA test with

Fischer's least significant difference post-test. For measuring cell viability after phytochemical exposure and the drug accumulation assays, growth/accumulation velocities and endpoint states were first calculated. The data were then analysed using a one-way ANOVA test with the inclusion of Fischer's least significant difference post-test. For the mutant selections, the following formula was used to calculate a mutation frequency for each pathogen against each tested phytochemical at $1 \times \text{MIC}$.

$$\text{Average mutation frequency} = \frac{[\text{Average CFU/mL for each inoculum}]}{\text{Corresponding DMSO viable count (CFU/mL)}}$$

For the phenotyping of thymol-selected mutants, the previously stated analyses for the measuring of cell viability after phytochemical exposure and the drug accumulation assays were performed. Crystal violet assays were analysed using a one-way ANOVA test with the inclusion of Fischer's least significant difference post-test, whereas congo red assays were quantitatively observed. Finally, five individual cells randomly selected from the TEM images were analysed via the ImageJ version 1.53r software (National Institutes of Health, MD, USA). Mean grey values were measured. Mean grey values were labelled as average cytoplasmic density values in the corresponding table, as this was the intended inference. Numerical values were displayed to two decimal places and a statistical analysis via a one-way repeated measures ANOVA test, with the inclusion of Fisher's LSD test, performed using the GraphPad software package version 8.0, to distinguish statistically significant results.

3. Results

3.1. Nine Compounds Show Consistent Antimicrobial Activity

All 14 compounds were screened for both direct antimicrobial activity and the ability to potentiate other antimicrobial agents, with most demonstrating activity in either assay. Examples of results from growth inhibition and potentiation assays are shown in Figure 1a,b, with full data in the Appendix (Tables A1–A4). Nine compounds showed a reproducible, statistically significant reduction in the average $\text{OD}_{(600 \text{ nm})}$ achieved by cultures of the four tested pathogens, although the spectrum of activity varied between compounds. Dose-dependent inhibition of growth was also observed; for example, the flavanone eriodictyol reduced the average $\text{OD}_{(600 \text{ nm})}$ measurements of *S. aureus* and *L. monocytogenes* cultures by 86.87% (at 0.5 mg/mL) and 12.31% (at 0.125 mg/mL), respectively, compared to the relevant controls (Figure 1a,b). Naringenin showed potent activity against *S. aureus* (reducing growth by 92.8%, 89.5%, and 8.9% at 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL, respectively). Thymol at 0.5 mg/mL showed a wide spectrum of activity and reduced the average $\text{OD}_{(600 \text{ nm})}$ of *S. Typhimurium*, *P. aeruginosa*, and *L. monocytogenes* by 48.82%, 42.59%, and 23.35%, respectively, although it was not active against *S. aureus*.

The data points show the final OD achieved after 16 h of incubation of four biological replicates, with three technical replicates each. The horizontal bars show the mean for each set. The dashed line shows the OD from media alone. Statistical analysis was performed using the GraphPad software v.8, using a one-way ANOVA test with Fischer's least significant difference test. The error bars indicate SEM (\pm).

PABN, a known efflux inhibitor, was used as a control to potentiate the activity of chloramphenicol (present at $0.25 \times \text{MIC}$ in all conditions).

To determine impacts on viability, the number of surviving cells was enumerated after exposure to two concentrations of the compounds that demonstrated activity in the initial optical density screens. Eight of the nine tested compounds exerted a significant reduction in viability numbers of at least one pathogen (Figures 2 and A1 and Tables A5–A8).

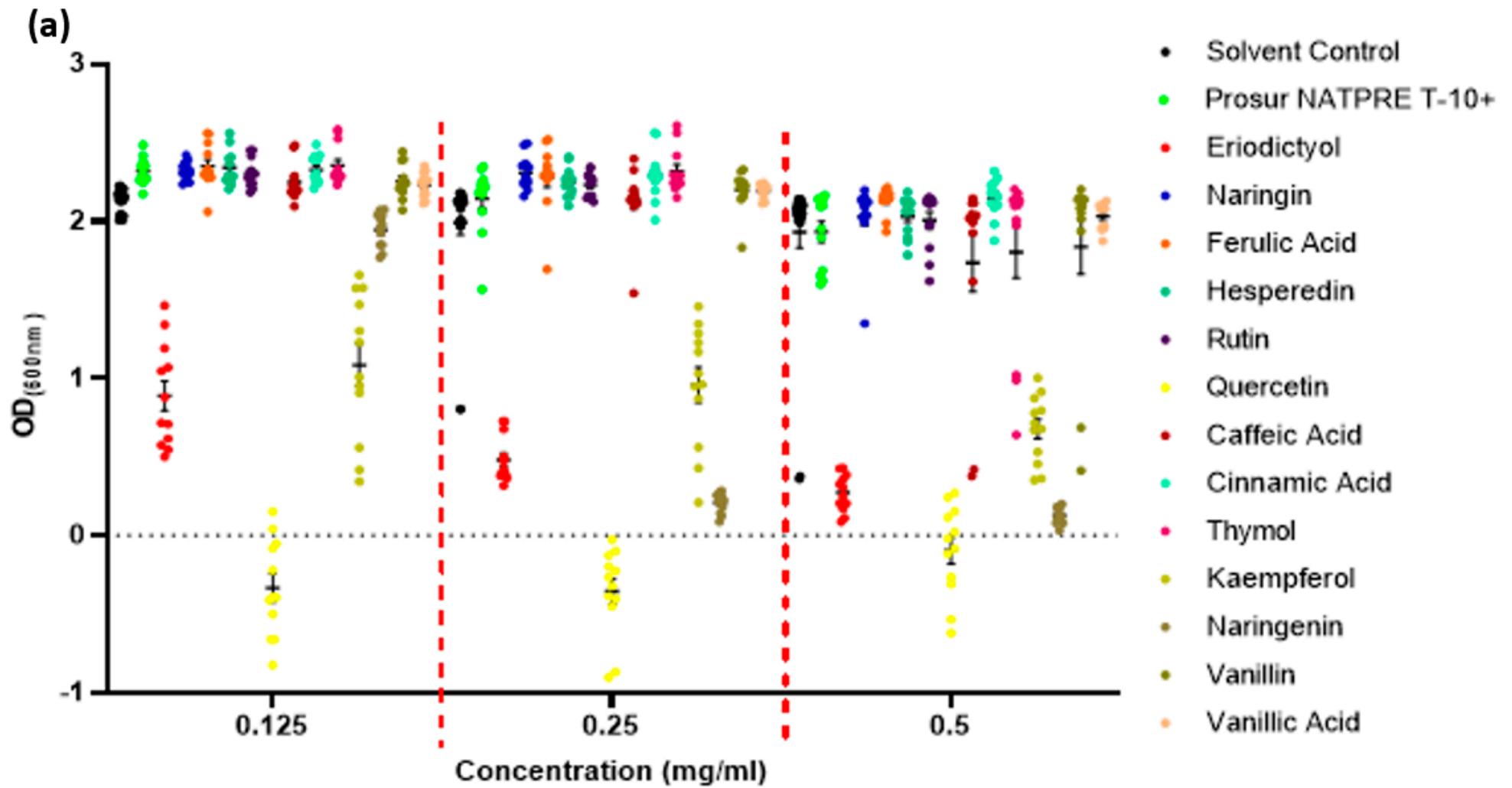


Figure 1. Cont.

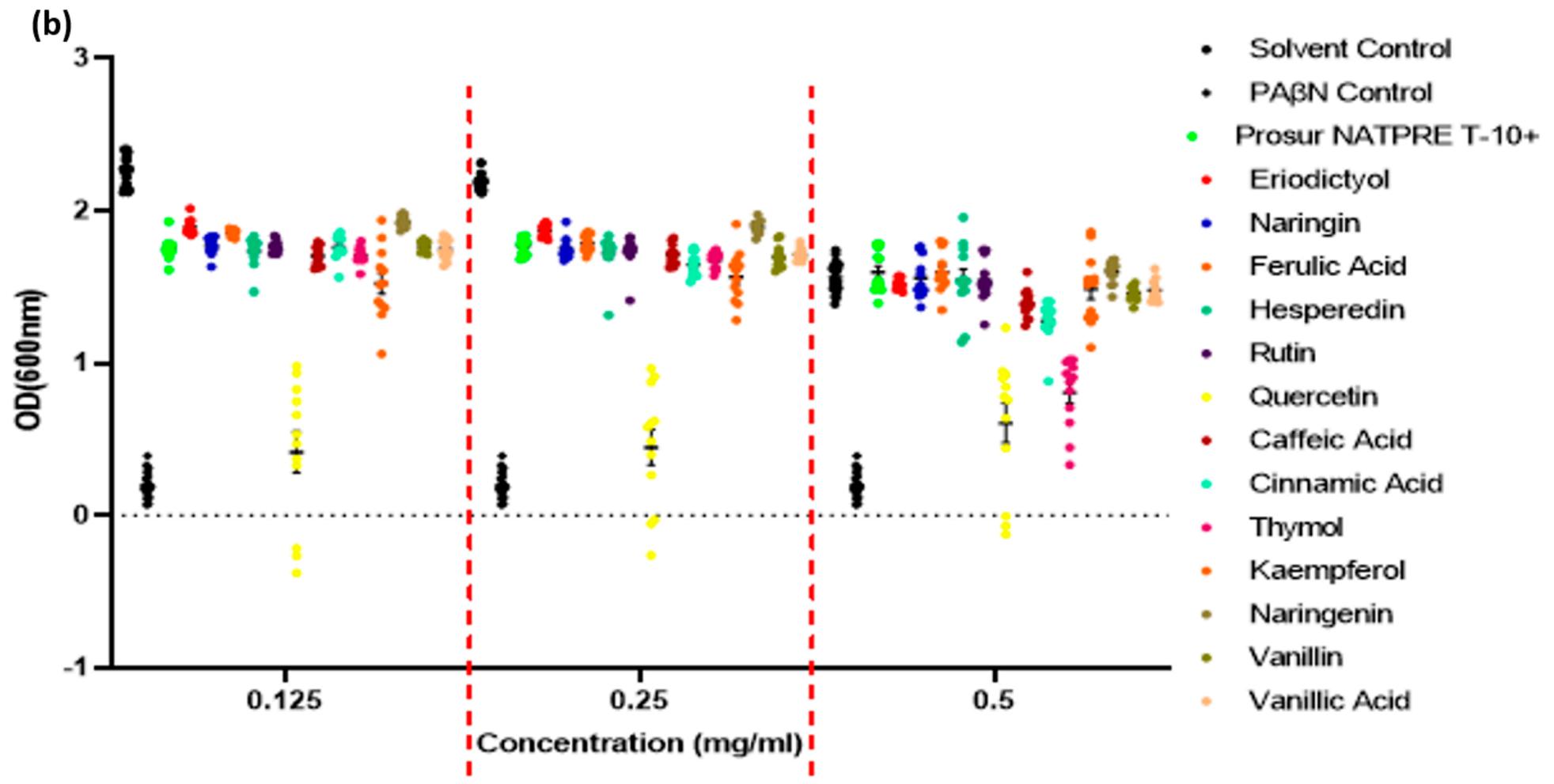


Figure 1. Example antibacterial and potentiating activity of phytochemicals. (a) Growth of *S. aureus* in the presence of different phytochemicals. (b) Impact of phytochemicals on growth of *S. Typhimurium* in the presence of a sub-MIC concentration of chloramphenicol. Red dotted lines included to visually segregate the tested concentrations.

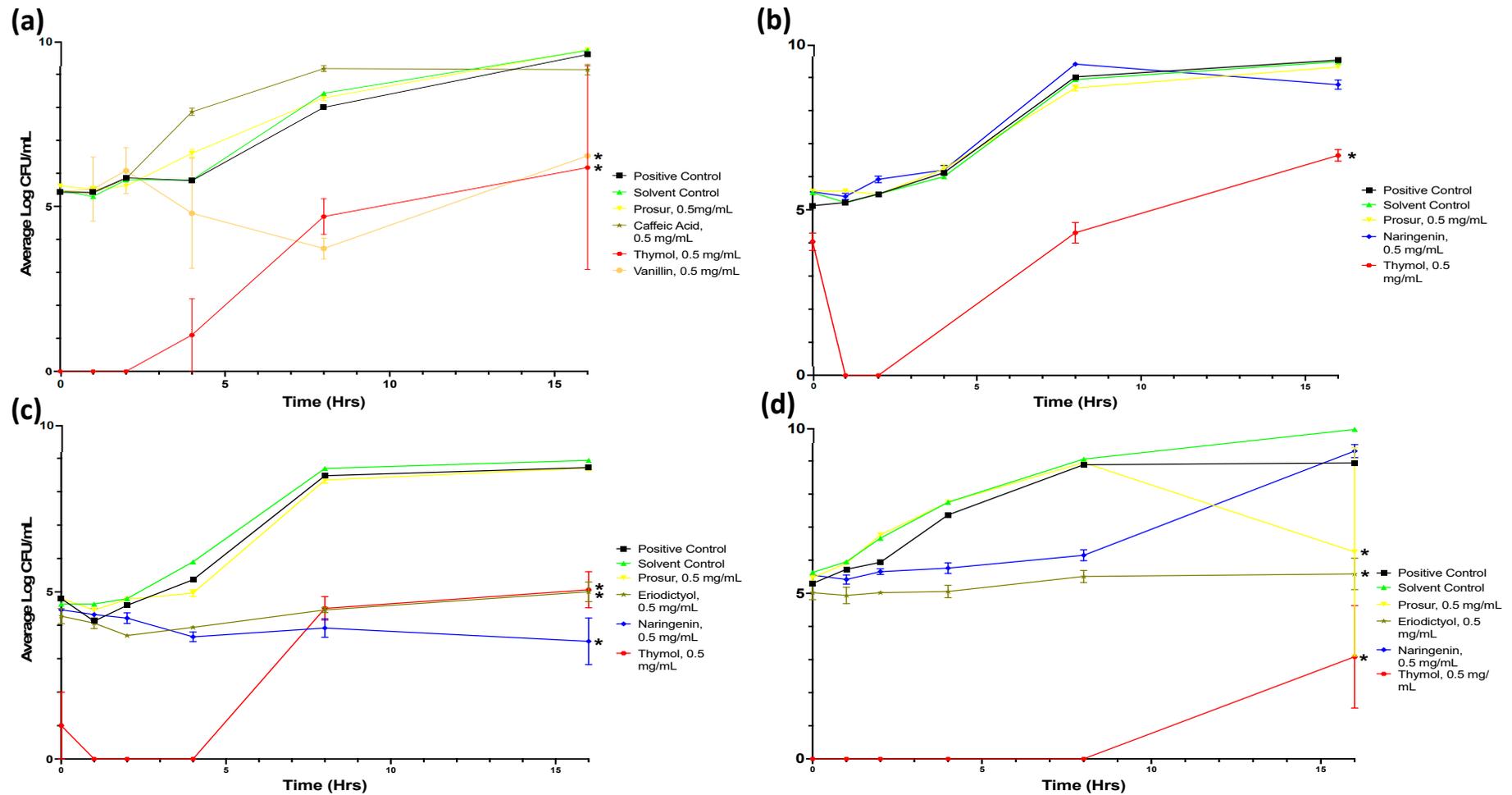


Figure 2. Viability of pathogens challenged with 0.5 mg/mL of selected phytochemicals. Viable numbers (based on CFU/mL) of the following: (a) *S. Typhimurium*, (b) *P. aeruginosa*, (c) *S. aureus*, and (d) *L. monocytogenes* following exposure to different phytochemicals. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Graphs display the averaged values of three technical replicates for three biological replicates. Error bars indicate SEM (\pm); asterisks denote statistical significance.

In general, the phytochemicals were more effective at inhibiting the growth of the Gram-positive than the Gram-negative pathogens tested, and dose-dependent effects were again observed.

Prosur NATPRE T-10+ significantly reduced the CFU/mL of *L. monocytogenes* cultures by 38.8% compared to the relevant control; however, this compound mixture did not have a significant inhibitive effect on any other tested pathogen (see Figure 2). Eriodictyol significantly decreased the growth velocity and endpoint states of *S. aureus* by 84.7% and 44.1%, respectively, and of *L. monocytogenes* by 61.2% and 35.3%, respectively (Figure 3). The flavonols quercetin and kaempferol both significantly decreased the growth velocities of *S. aureus* (quercetin and kaempferol) and *L. monocytogenes* (quercetin alone) cultures (Figure A1 and Tables A5–A8). The flavanone naringenin showed a statistically significant reduction in growth of all four pathogens (Figures 2 and A1 and Tables A5–A8). Thymol was significantly more active than any other compound and exerted the most consistent and potent inhibitive activity against all of the tested pathogens (Figure 2). This was particularly pronounced for earlier time points with decreased growth velocities for *S. Typhimurium* (70.2% reduction), *S. aureus* (75.9% reduction), *P. aeruginosa* (87.6% reduction), and *L. monocytogenes* (>99% reduction). The final number of viable cells achieved by each population was also lower after thymol exposure for *S. Typhimurium* (66.8% reduction), *S. aureus* (43.2% reduction), *P. aeruginosa* (29.5% reduction), and *L. monocytogenes* (70.2% reduction) compared to the appropriate controls.

The Prosur NATPRE T-10+ mix and vanillin exhibited dose-dependent effects on *L. monocytogenes*, with the former displaying inhibitive activity at 0.5 mg/mL but not at the lower concentration of 0.05 mg/mL and vice versa for the latter aldehyde compound (see Tables A8 and A9).

3.2. Caffeic Acid and Prosur NATPRE T-10+ Enhance Cellular Permeability

Drug accumulation after exposure to the phytochemicals was determined, and selected results are shown in Figure 3 (full data can be found in Figures A2–A5 and Tables A10–A13). Six compounds provoked a statistically significant increase in drug accumulation by *S. Typhimurium*, *P. aeruginosa*, and *L. monocytogenes* cultures. Caffeic acid (0.5 mg/mL) increased the velocity of resazurin accumulation within *S. Typhimurium* by 40.8% compared to the solvent vehicle control and provoked a smaller increase in *P. aeruginosa* cultures (Figures A2–A5, Tables A10–A13). The Prosur NATPRE T-10+ mixture was most effective at increasing the drug accumulation of all the tested pathogens, increasing the rate of resazurin accumulation by between 70 and 200% in *S. Typhimurium* (Figure 3) and *P. aeruginosa* (Figures A2–A5, Tables A10–A13).

Ferulic acid and naringenin were also able to increase resazurin accumulation, increasing the average fluorescence accumulation velocity of *S. Typhimurium* by 29.1% and 17.9%, respectively (Figure 3). For *S. aureus* and *L. monocytogenes* (Figure 3), ethidium bromide was used rather than resazurin as the fluorescent indicator due to these microorganisms' rapid metabolism of the latter compound. This showed increased accumulation after exposure to the Prosur NATPRE T-10+ mix in *S. aureus* (Figure 3).

3.3. Thymol Selects for Resistant Mutants at a Rate Similar to Classic Antibiotics

To determine the potential for resistance emergence, *S. Typhimurium*, *S. aureus*, *P. aeruginosa*, and *L. monocytogenes* were exposed to the MIC and 2× the MIC of caffeic acid, Prosur NATPRE T-10+, or thymol in agar. After inoculation of plates with large populations of each organism, no significant inhibition was observed from caffeic acid or the Prosur NATPRE T-10+ mix, and bacterial lawns of growth were observed. Thymol, however, was able to inhibit growth of the majority of populations and selected for discrete mutant colonies of *S. Typhimurium*, *S. aureus*, and *P. aeruginosa* at an average rate of 3.77×10^{-8} (Table 1).

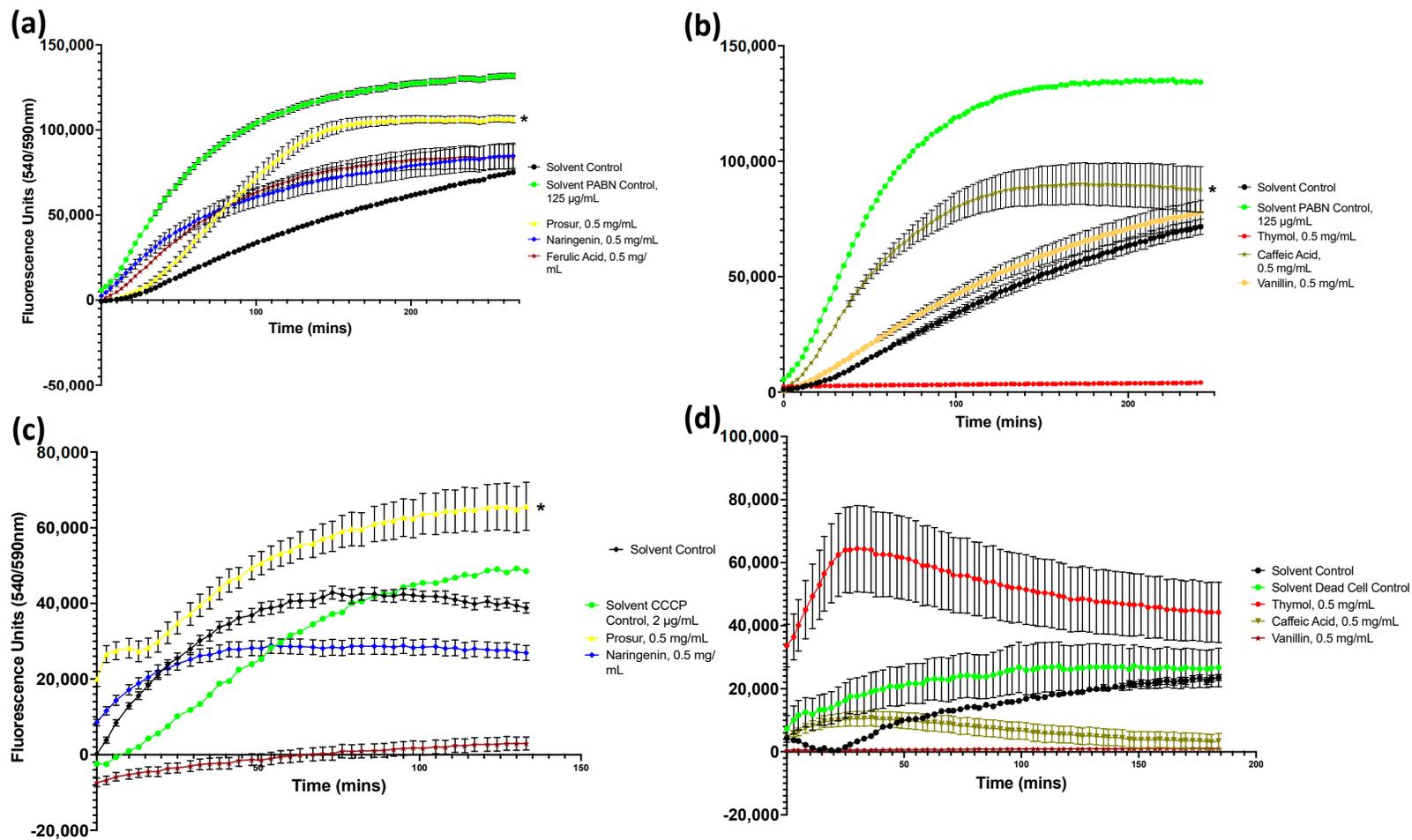


Figure 3. Selected resazurin accumulation assays for pathogens challenged with 0.5 mg/mL of various phytochemicals. (a) *S. Typhimurium* challenged with the Prosur NATPRE-T10+ mix, naringenin, and ferulic acid. (b) *S. Typhimurium* challenged with thymol, caffeic acid, and vanillin at 0.5 mg/mL. the Prosur NATPRE-T10+ mix, naringenin, and ferulic acid. (c) *S. aureus* challenged with the Prosur NATPRE-T10+ mix, naringenin, and ferulic acid. (d) *L. monocytogenes* challenged with thymol, caffeic acid, and vanillin. Points show the blank-adjusted and averaged values of five technical replicates for three biological replicates. Error bars indicate SEM (\pm); asterisks denote statistical significance.

Table 1. Frequency of selection of thymol-tolerant mutants.

Microorganism	Frequency of Mutant Selection
<i>S. Typhimurium</i>	6.84×10^{-9}
<i>S. aureus</i>	1.47×10^{-7}
<i>P. aeruginosa</i>	3.01×10^{-8}
<i>L. monocytogenes</i>	N/D *
Average for all species	3.77×10^{-8}

* N/D: not determined due to an absence of identifiable colonies.

3.4. Thymol-Selected Mutants of *S. Typhimurium* Display Tolerance Rather Than Resistance to Thymol

Eight randomly selected *S. Typhimurium* mutant colonies were characterised for thymol susceptibility; no strain grew at or above the MIC of 0.5 mg/mL, but at 0.25 mg/mL thymol the candidate mutant strains produced larger colonies than the parental strain (Figure A6).

3.5. *S. Typhimurium* Mutant Sequencing Reveal Efflux-Associated SNPs

The mutant strains of *S. Typhimurium* were sequenced, and a total of four SNPs were identified. *S. Typhimurium* mutant strain #2 was found to harbour a substitution within the putative class I SAM-dependent methyltransferase *yafS* gene, causing a H107Q substitution within the protein. This strain #2 also possessed a unique substitution within the *tetR/acrA* family transcriptional regulator *ramR* gene, resulting in the substitution of phenylalanine amino acid 48 of RamR to cysteine, a change not currently identified within the literature. *S. Typhimurium* mutant strain #1 contained another known mutation within *ramR*, resulting in a G96D amino acid residue substitution.

One final SNP identified within three mutants (#1, #5, and #6) was the insertion of a thymine base between STM14_18795 (a putative cytoplasmic protein, *glpF* homologue) and STM14_18790 encoding the putative DeoR family transcriptional regulator *glpR*.

3.6. Thymol-Tolerant *S. Typhimurium* Mutants Display Decreased Susceptibility to Antibiotics

As sequencing of the thymol-selected mutant strains revealed the presence of SNPs within loci associated with efflux activity, the antibiotic sensitivity of the strains was determined (Table 2). Mutant strains #1 and #2 demonstrated small increases in the MICs of tetracycline, ampicillin, chloramphenicol, and nalidixic acid, all antibiotics subject to efflux, which is consistent with the *ramR* mutations seen in these strains.

Table 2. Changes in antibiotic susceptibility of thymol-selected *S. Typhimurium* mutants. Parental strain MICs for the listed antibiotics, in order as presented, are as follows: 2.67 µg/mL, 1 µg/mL, 6 µg/mL, 3.33 µg/mL, and 4 µg/mL. A # denotes the allocated number of the mutant strain.

		Average MIC Fold Changes of Mutant Strains Relative to Parent				
		Antibiotic				
		Kan	Tet	Amp	Chl	Nal
<i>S. Typhimurium</i>	WT	1.00	1.00	1.00	1.00	1.00
	#1	1.00	2.67	4.21	3.42	4.17
	#2	0.83	2.67	4.21	3.42	4.17
	#6	1.00	1.67	2.38	1.67	2.00

Values show the average MIC fold changes for five antibiotic compounds (Kan = kanamycin, Tet = tetracycline, Amp = ampicillin, Chl = chloramphenicol, and Nal = nalidixic acid) against mutants of *S. Typhimurium* compared to the parental (WT) strain. Data are the average of three biological replicates (each with three technical replicates).

3.7. *S. Typhimurium* Mutant Growth in the Presence of Thymol

S. Typhimurium mutant strains #1 and #2 were further investigated via growth curves to quantify their growth kinetics under 0.25 mg/mL thymol challenge; the results of these experiments are presented in Figure 4. Thymol at 0.25 mg/mL reduced the growth of both the mutant strains and the parental *S. Typhimurium* strain, whereas all cultures grew with no significant differences if unchallenged (Figure A7). No statistically significant difference was seen between the mutants and the parent in growth in the presence of thymol.

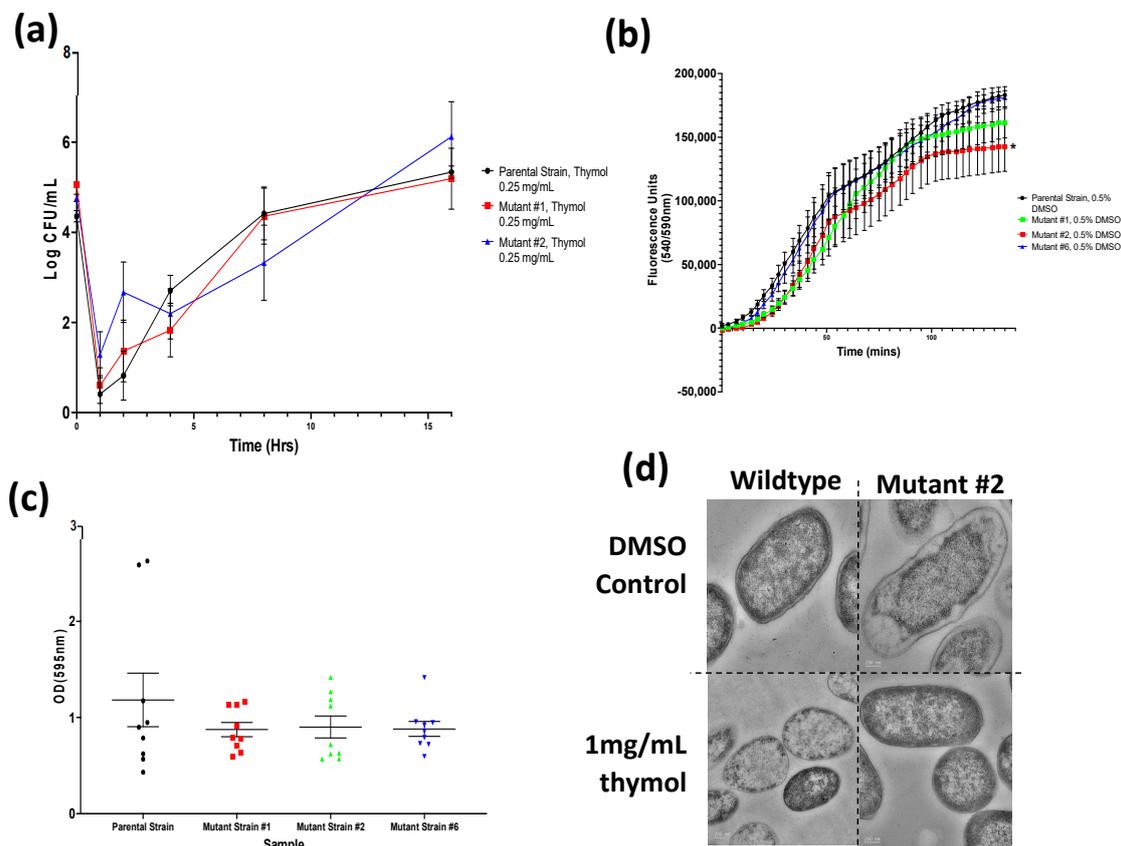


Figure 4. Characterisation of thymol-tolerant *S. Typhimurium* mutants. (a) Growth of parent and mutant strains in the presence of thymol (data show averages from nine replicates); asterisks denote statistical significance. (b) Resazurin accumulation assays (points show average values from five technical replicates of three biological replicates); asterisks denote statistical significance. (c) Biofilm biomass measured by crystal violet assays (points show average values from nine replicates). (d) *S. enterica* wild-type and mutant #2 TEM images under thymol and equivalent solvent vehicle exposure.

3.8. *S. Typhimurium* Mutants Accumulate Less Resazurin Than Parent Strain

Figure 4 depicts the accumulation of resazurin by selected *S. Typhimurium* strains and shows lower accumulations for both the *ramR* mutant *S. Typhimurium* strains #1 and #2 compared to the parental control (statistically significant for mutant #2 only). This is consistent with increased efflux activity.

3.9. *S. Typhimurium* Mutants Display a Decreased Biofilm Capacity

Crystal violet staining assays were performed to determine the thymol-tolerant mutants' biofilm-forming capacity. Figure 4 shows the biomass of biofilms produced by the parent and mutants. The thymol-tolerant *S. Typhimurium* mutants exhibited decreased biomass production compared to the parental strain. This trend was further observed in congo red plating, with a lesser degree of staining for the resulting colonies (Figure A8).

3.10. Thymol Exposure Induces Damage to the Bacterial Envelope

Transmission electron microscopy (TEM) was used to observe the morphological effects of thymol exposure on bacterial cells of *S. Typhimurium*. Figure 4 presents the morphologies of the parental and mutant #2 strains with and without exposure to 1 mg/mL thymol. The parental strain presented a classic envelope and bacillary morphology when unchallenged with thymol. After 2 h of exposure, the parental *S. Typhimurium* strain presented a significantly altered rod shape with a decreased cytoplasmic density and envelope thickness and a “fluffy” cellular surface (Figure 4, Table 3). In contrast, the efflux-associated mutant *S. Typhimurium* strain #2 presented a more rounded morphology with a thinner envelope and rougher surface even under control conditions. However, this morphology was largely unaltered when exposed to 1 mg/mL thymol (Figure 4, Table 3).

Table 3. Quantitative ImageJ analysis of TEM images from thymol-exposed *S. Typhimurium*. Values in bold denote statistically significant samples.

S. Typhimurium TEM Image Analysis				
Sample	Average Cytoplasmic Density	SEM (\pm)	p-Value	
WT control	128.2	1.92	-	
WT solvent control	98.6	8.66	0.0045	
WT 1 mg/mL thymol	140.5	5.96	0.1986	
<i>S. enterica</i> #2 control	145.5	2.66	-	
<i>S. enterica</i> #2 solvent Control	139.8	1.90	0.0912	
<i>S. enterica</i> #2 1 mg/mL Thymol	128.5	1.26	<0.0001	

Quantitative values produced through analysis of five randomly selected cells across two TEM images are presented above. Average cytoplasmic density relates to mean grey values; the higher the value, the less dense the cytoplasmic contents of the analysed cells. Values presented at two decimal places. Statistical analysis was performed using GraphPad software v.8 using a one-way repeated measures ANOVA Test with Fischer’s LSD test. Values in bold are statistically significant compared to the relevant control.

4. Discussion

The initial panel of 14 phytochemicals screened for antimicrobial activity against food-borne pathogens was chosen considering previous reports of activity and availability; of these, nine showed some activity. Previous work has suggested hesperidin [32], rutin [33], and vanillic acid [34] to have antimicrobial activity, but this was not observed at any tested concentration against any pathogen tested here. Of the nine phytochemicals selected for further testing, seven displayed a capacity to inhibit the growth of at least one microorganism tested. Naringenin at a concentration of 0.5 mg/mL did not present significant activity against *S. Typhimurium*, corroborating other works that present an MIC of 1 mg/mL [35], although it did exert a significant reduction in $OD_{(600\text{ nm})}$ measurements against *S. aureus*, adding to earlier work in the field that has found this to be the compounds’ MIC [36]. Quercetin, although unsuitable for the OD-based assays devised here due to its natural yellow pigmentation, has previous evidence supporting its antimicrobial capacity [37] and was thus carried forward for further investigation. Kaempferol was also selected on a similar basis [38]. Other compounds from the selected nine have also had previous studies reporting on their antimicrobial activity, such as eriodictyol [39]. Thymol, however, was the most potent phytochemical and showed consistent bioactivity against the panel of organisms, which supports previous studies reporting MIC ranges of 0.005–0.662 mg/mL against these organisms [40–44]. Alternative evidence supporting the antimicrobial activity of thymol suggests an MIC of 0.25 mg/mL against *S. aureus* and a minimum bactericidal concentration (MBC) of 0.5 mg/mL [45]. In contrast we observed an increase in CFU/mL from under the detection threshold after eight hours of incubation in the presented growth curves, suggesting that, although 0.5 mg/mL may be the MIC, the MBC under these con-

ditions may in fact be higher due to various environmental, methodological, and genetic factors. Research to improve the solubility and stability of antimicrobial phytochemicals, including the nanocomplexation and production of thymol composites, have also shown an increase in antimicrobial activity against many foodborne pathogens [45,46].

In addition to direct antibacterial activity, there is potential for phytochemicals to be deployed in combination with other antimicrobials [47–50]. Although checkerboard assays have traditionally been utilised for this end and remain a promising methodological avenue for future research, drug accumulation assays were used to infer synergistic capacities via the phytochemical's action of permeabilising the bacterial cell within the present work. Four of the selected nine phytochemicals (caffeic acid, ferulic acid, naringenin, and vanillin) displayed an ability to increase accumulation of resazurin/ethidium bromide, suggesting applications as potentiating agents [23,49,51–59]. Caffeic acid's potential to synergise with conventional antimicrobials has been described previously against *E. coli*, *P. aeruginosa*, and *S. aureus* strains [47,60,61]. We extend these data to now include *S. Typhimurium*. In contrast, researchers have published data providing evidence that thymol possesses synergistic effects [42,62–64], including Miladi H. et al. (2016) [65], who employed a similar methodology in the ethidium bromide accumulation assay. The authors found that thymol (in addition to the related compound carvacrol) at 0.5 mg/mL inhibited the efflux of ethidium bromide from cells of *S. aureus*, *E. coli*, *S. Typhimurium*, and *Salmonella enteritidis* [65]. We did not predict this conclusion from our data, potentially because thymol was found to be rapidly bactericidal in our experiments, making synergies hard to detect. In addition, there are differences in the employed methodologies; we used drug accumulation assays to directly identify compounds that were likely to impact membrane permeability or inhibit efflux. Other work has relied on checkerboard assays, which identify synergies but provide no mechanistic information.

Few studies to date have investigated the potential of phytochemicals to select for resistant populations. To explore this, we determined the frequency of mutant selection for *S. Typhimurium*, *S. aureus*, *P. aeruginosa*, and *L. monocytogenes* when exposed to caffeic acid, thymol, and the Prosur NATPRE T-10+ mix. Of these, caffeic acid and the Prosur NATPRE T-10+ mix were unable to inhibit bacterial growth on agar plates when challenged with the large inocula used for mutant selection. Thymol, however, did select for mutant colonies at an average rate of 3.77×10^{-8} across the four pathogens tested. This mutation frequency was similar (approximately $\sim 10^{-8}$ to 10^{-9} [66–68]) to that found for multiple bacterial species, including the common foodborne pathogens tested here, when challenged with classical antibiotics such as quinolones [66], fluoroquinolones [68], and norfloxacin [67].

The *S. Typhimurium* mutants demonstrated a mild level of tolerance to thymol itself, with no MIC change, but evidenced improved growth in the presence of thymol. Two of the mutants also showed decreased susceptibility to antibiotics (tetracycline, ampicillin, chloramphenicol, and nalidixic acid), reduced accumulation of the efflux substrate resazurin, and lower biofilm biomass production (Figure 4). Sequencing of the thymol-selected *S. Typhimurium* mutants revealed the presence of SNPs within the *yafS* gene [69,70], not characterised in detail for *S. Typhimurium*, and upstream of *glpR*, a repressor of the sugar/carbohydrate transport and metabolism *glp* operon. Perhaps more relevant, however, were the multiple SNPs within the AcrAB efflux pump transcriptional repressor *ramR* [71] identified within different mutants. RamR is a well-known repressor of RamA, which in turn controls expression of many genes, including the *acrAB* multidrug efflux system [72]. Similar work with *E. coli* [73] selected a thymol-resistant strain (JM109-Thyr) via repeated exposure to sub-lethal thymol concentrations. Resulting isolates demonstrated two-fold increased thymol MICs and had mutations in *acrR*, the local repressor of *acrAB* [73]. Our data further suggest that thymol can select for mutants with decreased susceptibility and that they are efflux over-expression mutants (via *ramR* rather than *acrR* in *Salmonella*). These mutants demonstrate a classic efflux phenotype of low-level multidrug resistance, further supported by the decreased capacity for biofilm production presented in this work, which may have implications for the selection of collateral resistance following thymol

exposure. This potential for the selection of phytochemical and cross-resistance in bacterial pathogens is an important line of scientific enquiry with consequences for the practical adoption of phytochemicals as substitute antimicrobials in the clinic, farm, household, or food industries. The latter is particularly true given the push for non-chemical food preservatives [74,75] and the status of phytochemicals as generally recognised as safe (GRAS) for inclusion in food products [76]. If antimicrobial phytochemicals are to be employed en masse, the possibility of bacterial resistance to these and extraneous compounds evolving along the same lines as the current AMR crisis must be considered.

Finally, the identification of efflux-associated SNPs within the thymol-selected mutants prompted the application of TEM to observe the effect of thymol challenge on the cell envelope morphology of *S. Typhimurium*. Here, we observed significant envelope damage and cytoplasmic shrinkage of the parental strain, with a greater robustness exhibited by the thymol-tolerant mutant when exposed to thymol (Figure 4, Table 3). This disruption of the bacterial envelope/membrane is in line with other studies that observed similar results both in *S. enterica* and in other bacterial species [77–81] and supports previous suggestions for interactions with the bacterial envelope as mediating the antimicrobial mechanism of action for thymol. Although there is a strong evidence base to support an envelope-targeting mechanism for thymol, the TEM imaging implemented within this study only provides a semi-qualitative observation. Future work may focus on the use of scanning electron microscopy to better observe the envelope surface and assays designed to investigate the impact of the observed effects on cell viability and cellular functions. There have also been, however, multiple mechanisms of antimicrobial action reported for this monoterpene phenol. There are numerous reports supporting the inhibition of efflux pumps, the disruption of natural biofilm functions, the inhibition of motility and key bacterial enzymes [82], and the dysregulation of protein and DNA synthesis [75]. This phenomenon of multiple inhibitive mechanisms is not unique to thymol, as it appears that many phytochemicals share a multi-pronged paradigm of antibacterial activity [75].

5. Conclusions

We have shown that various phytochemicals have antimicrobial effects against important foodborne pathogens, that this differs between compounds and species, and that some phytochemicals also demonstrate good promise as potentiators/synergisers in combination with other antimicrobial agents. Thymol was the most active compound tested, and although we were able to select for mutants with decreased susceptibility to thymol, they were efflux mutants, which has implications for thymol application, as these mutants also displayed a decreased susceptibility to antibiotics. Although phytochemicals represent an alternative source of directly and synergistic antimicrobial compounds to be exploited, further research is needed into their mechanisms of action and potential for selection of resistance.

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Data Availability Statement: Data is contained within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Summary of results of the growth inhibition assays against Gram-negative species.

Gram-Negative Pathogen OD _(600 nm) after Polyphenol Exposure as a Percentage of Solvent Controls							
Phytochemical	Concentration (mg/mL)	<i>S. Typhimurium</i>			<i>P. aeruginosa</i>		
		OD _(600 nm)	<i>p</i> -Value	SEM (±)	OD _(600 nm)	<i>p</i> -Value	SEM (±)
Prosur	0.5	103.01	0.3594	0.03	106.07	0.3583	0.07
	0.25	106.26	0.0004	0.02	100.99	0.4665	0.03
	0.125	106.43	0.0002	0.02	101.87	0.4057	0.05
Eriodictyol	0.5	103.48	0.2970	0.03	109.58	0.1476	0.05
	0.25	103.25	0.0571	0.02	102.22	0.1080	0.01
	0.125	104.92	0.0042	0.02	103.09	0.1741	0.03
Naringin	0.5	101.28	0.7033	0.03	106.58	0.3198	0.07
	0.25	105.03	0.0038	0.01	104.13	0.0037	0.02
	0.125	107.18	<0.0001	0.02	103.91	0.0859	0.04
Ferulic acid	0.5	84.23	<0.0001	0.05	89.97	0.1301	0.10
	0.25	102.70	0.1101	0.02	102.18	0.1159	0.02
	0.125	106.65	0.0001	0.02	104.52	0.0475	0.04
Hesperidin	0.5	98.03	0.5523	0.07	110.10	0.1279	0.06
	0.25	102.74	0.1072	0.05	103.04	0.0307	0.02
	0.125	104.30	0.0117	0.05	103.18	0.1643	0.04
Rutin	0.5	97.74	0.4925	0.02	104.79	0.4648	0.07
	0.25	105.17	0.0030	0.02	103.18	0.0240	0.02
	0.125	106.69	0.0001	0.02	103.83	0.0917	0.03
Quercetin	0.5	21.07	-	-	48.83	-	-
	0.25	22.87	-	-	44.82	-	-
	0.125	23.64	-	-	45.37	-	-
Caffeic acid	0.5	96.19	0.3627	0.04	94.87	0.3005	0.04
	0.25	105.08	0.0107	0.04	104.81	0.0006	0.02
	0.125	106.07	0.0018	0.03	104.09	0.0165	0.02
Cinnamic acid	0.5	82.39	<0.0001	0.04	79.82	<0.0001	0.06
	0.25	103.48	0.0767	0.04	100.18	0.8788	0.02
	0.125	106.03	0.0019	0.03	102.22	0.1899	0.02
Thymol	0.5	51.18	<0.0001	0.09	57.41	<0.0001	0.07
	0.25	106.26	0.0018	0.03	103.27	0.0159	0.02
	0.125	108.42	<0.0001	0.03	104.57	0.0080	0.03
Kaempferol	0.5	83.69	-	-	86.34	-	-
	0.25	86.28	-	-	91.15	-	-
	0.125	85.42	-	-	90.78	-	-
Naringenin	0.5	101.25	0.7656	0.04	95.43	0.3555	0.05
	0.25	103.89	0.0490	0.04	101.50	0.2604	0.03
	0.125	106.25	0.0013	0.03	102.52	0.1333	0.03
Vanillin	0.5	79.20	<0.0001	0.05	72.19	<0.0001	0.08
	0.25	100	0.9953	0.02	98.37	0.2299	0.02
	0.125	102.17	0.2556	0.03	102.57	0.1279	0.03
Vanillic acid	0.5	91.25	0.0373	0.03	81.49	0.0003	0.09
	0.25	99.59	0.8344	0.02	102.27	0.0941	0.02
	0.125	102.17	0.2581	0.02	104.48	0.0088	0.03

Data show the average OD_(600 nm) achieved after 16 h by four biological replicates, with three technical replicates each. Statistical analysis was performed using GraphPad software v.8, using a one-way ANOVA test with Fischer's least significant difference test. Values in bold denote statistically significant samples.

Table A2. Summary of results of the growth inhibition assays against Gram-positive species.

Gram-Positive Pathogen OD _(600 nm) after Polyphenol Exposure as a Percentage of Solvent Controls							
Phytochemical	Concentration (mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
		OD _(600 nm)	<i>p</i> -Value	SEM (±)	OD _(600 nm)	<i>p</i> -Value	SEM (±)
Prosur	0.5	93.20	0.0317	0.07	91.08	0.2676	0.08
	0.25	107.79	0.0628	0.06	104.97	0.3098	0.06
	0.125	108.47	0.0049	0.02	106.29	0.1934	0.07
Eriodictyol	0.5	13.13	<0.0001	0.03	87.84	0.1321	0.12
	0.25	24.16	<0.0001	0.04	93.38	0.1795	0.13
	0.125	41.59	<0.0001	0.09	87.69	0.0118	0.10
Naringin	0.5	97.97	0.5192	0.06	93.19	0.3982	0.09
	0.25	115.93	0.0002	0.03	103.20	0.5112	0.06
	0.125	108.61	0.0043	0.02	104.81	0.3160	0.07
Ferulic acid	0.5	102.80	0.3778	0.02	94.76	0.5139	0.09
	0.25	114.62	0.0007	0.06	108.89	0.0712	0.08
	0.125	110.07	0.0009	0.04	107.12	0.1405	0.07
Hesperidin	0.5	97.88	0.4930	0.04	89.35	0.1857	0.18
	0.25	112.91	0.0025	0.03	97.13	0.5583	0.08
	0.125	109.50	0.0017	0.03	94.05	0.2170	0.13
Rutin	0.5	96.67	0.2857	0.05	93.73	0.4368	0.06
	0.25	112.11	0.0043	0.02	107.50	0.1281	0.08
	0.125	108.01	0.0077	0.02613	105.23	0.2771	0.07
Quercetin	0.5	−4.57	-	-	10.19	-	-
	0.25	−17.87	-	-	28.36	-	-
	0.125	−15.60	-	-	35.90	-	-
Caffeic acid	0.5	97.20	0.7960	0.1843	88.86	0.2032	0.09
	0.25	107.29	0.0645	0.05955	96.17	0.4586	0.05
	0.125	105.24	0.0088	0.03398	97.12	0.5536	0.06
Cinnamic acid	0.5	120.28	0.0641	0.03617	101.14	0.8942	0.11
	0.25	115.33	0.0002	0.04502	104.13	0.4269	0.08
	0.125	108.90	<0.0001	0.02623	104.85	0.3181	0.08
Thymol	0.5	101.01	0.9279	0.1631	76.65	0.0088	0.16
	0.25	116.53	<0.0001	0.04039	102.53	0.6218	0.06
	0.125	110.21	<0.0001	0.03669	106.74	0.1673	0.08
Kaempferol	0.5	37.98	-	-	20.30	-	-
	0.25	48.16	-	-	33.36	-	-
	0.125	50.70	-	-	27.03	-	-
Naringenin	0.5	7.204	<0.0001	0.01476	118.59	0.0353	0.14
	0.25	10.47	<0.0001	0.01797	108.13	0.1184	0.14
	0.125	91.06	<0.0001	0.03105	103.90	0.4215	0.11
Vanillin	0.5	102.91	0.7894	0.1753	98.05	0.8256	0.10
	0.25	113.84	0.0091	0.03674	109.27	0.0765	0.10
	0.125	110.20	0.0051	0.03132	107.05	0.1485	0.10
Vanillic acid	0.5	104.49	0.2035	0.02422	113.57	0.1211	0.10
	0.25	113.84	0.0106	0.01306	112.73	0.0158	0.10
	0.125	110.20	0.0242	0.02254	108.60	0.0786	0.10

Data show the average OD_(600 nm) achieved after 16 h by four biological replicates, with three technical replicates each. Statistical analysis was performed using GraphPad software v.8, using a one-way ANOVA test with Fischer's least significant difference test. Values in bold denote statistically significant samples.

Table A3. Summary of the growth potentiation assays with phytochemicals and 2 µg/mL chloramphenicol against Gram-negative species.

Gram-Negative Pathogens OD _(600 nm) Potentiative Assay Optical Densities, Percentage of Solvent Controls							
Phytochemical	Concentration (mg/mL)	<i>S. Typhimurium</i>			<i>P. aeruginosa</i>		
		OD _(600 nm)	<i>p</i> -Value	SEM (±)	OD _(600 nm)	<i>p</i> -Value	SEM (±)
PaβN-positive control	0.512	13.95	<0.0001	0.02	18.74	<0.0001	0.01
	0.5	103.57	0.3124	0.04	98.20	0.5309	0.02
Prosur	0.25	80.89	<0.0001	0.02	81.58	<0.0001	0.03
	0.125	77.62	<0.0001	0.02	77.16	<0.0001	0.03
Eriodictyol	0.5	98.64	0.6938	0.01	101.10	0.6932	0.04
	0.25	85.37	<0.0001	0.01	85.48	<0.0001	0.03
	0.125	83.91	<0.0001	0.01	81.64	<0.0001	0.02
Naringin	0.5	101.04	0.7756	0.04	94.27	0.0463	0.04
	0.25	80.06	<0.0001	0.02	81.08	<0.0001	0.04
	0.125	78.37	<0.0001	0.02	77.47	<0.0001	0.03
Ferulic acid	0.5	103.70	0.2932	0.04	99.68	0.9135	0.03
	0.25	81.53	<0.0001	0.02	81.17	<0.0001	0.03
	0.125	82.09	<0.0001	0.01	79.12	<0.0001	0.03
Hesperidin	0.5	100.45	0.8964	0.07	92.08	0.0062	0.04
	0.25	78.65	<0.0001	0.04	78.86	<0.0001	0.03
	0.125	76.91	<0.0001	0.03	77.51	<0.0001	0.04
Rutin	0.5	100.13	0.9658	0.04	96.20	0.1855	0.02
	0.25	79.24	<0.0001	0.03	79.17	<0.0001	0.04
	0.125	78.24	<0.0001	0.01	76.73	<0.0001	0.03
Quercetin	0.5	39.29	-	-	40.78	-	-
	0.25	20.45	-	-	21.57	-	-
	0.125	18.44	-	-	19.48	-	-
Caffeic acid	0.5	87.87	0.0002	0.03	86.92	0.0003	0.04
	0.25	78.28	<0.0001	0.02	81.13	<0.0001	0.03
	0.125	75.44	<0.0001	0.02	81.86	<0.0001	0.04
Cinnamic acid	0.5	80.42	<0.0001	0.04	86.98	0.0003	0.03
	0.25	75.22	<0.0001	0.02	82.71	<0.0001	0.01
	0.125	77.97	<0.0001	0.02	82.69	<0.0001	0.03
Thymol	0.5	50.82	<0.0001	0.07	42.20	<0.0001	0.07
	0.25	76.50	<0.0001	0.02	82.40	<0.0001	0.02
	0.125	75.62	<0.0001	0.02	82.86	<0.0001	0.03
Kaempferol	0.5	93.75	-	-	86.67	-	-
	0.25	71.56	-	-	80.58	-	-
	0.125	67.51	-	-	71.38	-	-
Naringenin	0.5	100.76	0.8085	0.02	97.59	0.4949	0.03
	0.25	86.47	<0.0001	0.01	85.39	<0.0001	0.04
	0.125	85.42	<0.0001	0.01	82.08	<0.0001	0.04
Vanillin	0.5	92.04	0.0117	0.01	91.73	0.0197	0.02
	0.25	77.50	<0.0001	0.02	79.45	<0.0001	0.03
	0.125	78.24	<0.0001	0.01	79.34	<0.0001	0.02
Vanillic acid	0.5	93.30	0.0329	0.02	92.97	0.0471	0.03
	0.25	78.33	<0.0001	0.01	83.76	<0.0001	0.03
	0.125	77.44	<0.0001	0.02	81.91	<0.0001	0.03

Data show the average OD_(600 nm) achieved after 16 h by four biological replicates, with three technical replicates each. Statistical analysis was performed using GraphPad software v.8, using a one-way ANOVA test with Fischer's least significant difference test. Values in bold denote statistically significant samples.

Table A4. Summary of the growth potentiation assays with phytochemicals and 2 µg/mL chloramphenicol against Gram-negative species.

Gram-Positive Pathogens OD _(600 nm) Potentiative Assay Optical Densities, Percentage of Solvent Controls							
Phytochemical	Concentration (mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
		OD _(600 nm)	<i>p</i> -Value	SEM (±)	OD _(600 nm)	<i>p</i> -Value	SEM (±)
PaβN-positive control	0.512	5.79	<0.0001	0.01	−8.33	<0.0001	0.10
	0.5	17.85	<0.0001	0.03	108.54	0.1263	0.06
Prosur	0.25	19.50	<0.0001	0.05	100.55	0.9260	0.10
	0.125	54.26	<0.0001	0.13	96.74	0.6431	0.14
Eriodictyol	0.5	10.41	<0.0001	0.01	90.54	0.0915	0.07
	0.25	9.41	<0.0001	0.01	90.48	0.1138	0.07
	0.125	13.81	<0.0001	0.06	89.55	0.1370	0.11
Naringin	0.5	116.92	0.1361	0.12	105.89	0.2878	0.05
	0.25	90.95	0.0187	0.02	102.91	0.6277	0.11
	0.125	82.82	<0.0001	0.02	97.69	0.7414	0.15
Ferulic acid	0.5	95.37	0.6795	0.11	109.95	0.0750	0.05
	0.25	86.93	0.0008	0.06	105.18	0.3870	0.12
	0.125	80.06	<0.0001	0.05	101.44	0.8388	0.14
Hesperidin	0.5	81.25	0.0980	0.13	106.38	0.2502	0.10
	0.25	86.38	0.0005	0.04	96.63	0.5751	0.10
	0.125	79.26	<0.0001	0.05	92.99	0.3179	0.13
Rutin	0.5	96.73	0.7700	0.18	111.89	0.0339	0.08
	0.25	84.22	<0.0001	0.05	105.69	0.3413	0.13
	0.125	74.53	<0.0001	0.06	100.68	0.9231	0.16
Quercetin	0.5	9.34	-	-	44.61	-	-
	0.25	−36.27	-	-	56.81	-	-
	0.125	−35.57	-	-	59.77	-	-
Caffeic acid	0.5	0.580	<0.0001	0.01	101.19	0.8888	0.09
	0.25	2.15	<0.0001	0.02	100.97	0.8795	0.11
	0.125	2.45	<0.0001	0.02	97.31	0.7074	0.14
Cinnamic acid	0.5	84.64	0.1805	0.12	101.78	0.8364	0.13
	0.25	90.60	0.1190	0.05	105.18	0.4193	0.14
	0.125	80.43	0.0184	0.11	101.25	0.8622	0.16
Thymol	0.5	32.05	<0.0001	0.13	86.49	0.1251	0.20
	0.25	80.55	0.0016	0.10	100.76	0.9069	0.10
	0.125	73.78	0.0018	0.11	97.77	0.7555	0.16
Kaempferol	0.5	26.20	-	-	32.62	-	-
	0.25	20.99	-	-	21.45	-	-
	0.125	21.12	-	-	16.51	-	-
Naringenin	0.5	4.41	<0.0001	0.01	108.43	0.3349	0.10
	0.25	22.71	<0.0001	0.13	103.16	0.6209	0.10
	0.125	44.14	<0.0001	0.24	100.57	0.9377	0.12
Vanillin	0.5	62.72	0.0015	0.17	106.49	0.4586	0.10
	0.25	81.56	0.0027	0.09	103.79	0.5505	0.12
	0.125	74.20	0.0022	0.13	99.47	0.9391	0.15
Vanillic acid	0.5	56.74	0.0003	0.18	111.51	0.1879	0.10
	0.25	81.31	0.0023	0.07	101.64	0.7967	0.12
	0.125	70.32	0.0005	0.14	96.89	0.6627	0.15

Data show the average OD_(600 nm) achieved after 16 h by four biological replicates, with three technical replicates each. Statistical analysis was performed using GraphPad software v.8, using a one-way ANOVA test with Fischer's least significant difference Test. Values in bold denote statistically significant samples.

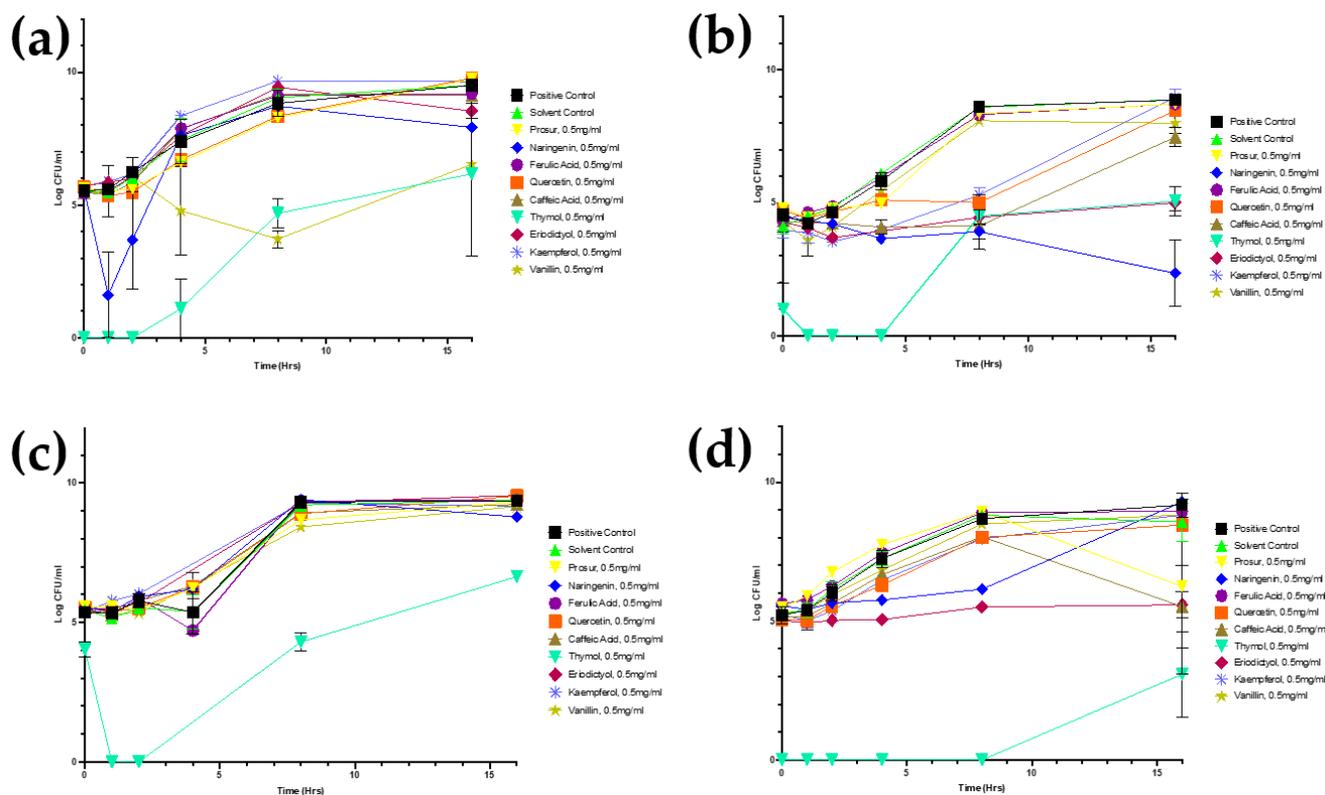


Figure A1. Growth curves of all tested microorganisms challenged with 0.5 mg/mL of all nine tested phytochemicals. Viable numbers (based on CFU/mL) of the following: (a) *S. Typhimurium*, (b) *S. aureus*, (c) *P. aeruginosa*, and (d) *L. monocytogenes* following exposure to different phytochemicals. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Graphs display the averaged values of three technical replicates for three biological replicates. Error bars indicate SEM (\pm).

Table A5. Summary of growth curves challenging the Gram-negative pathogens with various phytochemicals at 0.5 mg/mL concentrations.

Gram-Negative Pathogens Growth Curve Average Growth Velocities (%)						
Phytochemical (0.5 mg/mL)	<i>S. Typhimurium</i>			<i>P. aeruginosa</i>		
	Normalised Growth Velocity (CFU/mL/min)	SEM (\pm)	<i>p</i> -Value	Normalised Growth Velocity (CFU/mL/min)	SEM (\pm)	<i>p</i> -Value
Solvent control	100	0.08	-	100	0.73	-
Prosur	98.20	0.08	0.8981	102.86	0.07	0.8000
Eriodictyol	79.10	1.00	0.1385	114.09	0.07	0.2135
Ferulic acid	75.89	0.96	0.0883	89.87	0.99	0.3705
Quercetin	89.37	0.75	0.4486	106.83	0.04	0.5446
Caffeic acid	96.50	0.08	0.8026	96.08	0.70	0.7281
Thymol	29.80	0.27	<0.0001	12.41	0.32	<0.0001
Kaempferol	104.51	0.06	0.7482	112.78	0.07	0.2584
Naringenin	42.56	1.12	<0.0001	114.99	0.07	0.1859
Vanillin	85.28	0.72	0.2952	98.66	0.06	0.9053

Data presented are the average values as percentages of the controls for the calculated growth velocity (CFU/mL/min) metric. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Values in bold denote statistically significant samples.

Table A6. Summary of growth curves challenging the Gram-positive pathogens with various phytochemicals at 0.5 mg/mL concentrations.

Gram-Positive Pathogens Growth Curve Average Growth Velocities (%)						
Phytochemical (0.5 mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
	Normalised Growth Velocity (CFU/mL/min)	SEM (±)	<i>p</i> -Value	Normalised Growth Velocity (CFU/mL/min)	SEM (±)	<i>p</i> -Value
Solvent control	100	0.08	-	100	0.08	-
Prosur	85.48	0.63	0.1563	102.31	0.06	0.7756
Eriodictyol	15.32	0.29	<0.0001	38.76	0.31	<0.0001
Ferulic acid	86.26	0.64	0.1793	101.51	0.07	0.8530
Quercetin	30.18	0.36	<0.0001	59.70	0.91	<0.0001
Caffeic acid	20.92	0.44	<0.0001	78.43	0.60	0.0086
Thymol	24.12	0.36	<0.0001	0	0.00	<0.0001
Kaempferol	43.11489362	0.1693	<0.0001	86.40903881	0.04408	0.0940
Naringenin	1.925106383	0.1131	<0.0001	51.95677092	0.1677	<0.0001
Vanillin	81.95744681	0.6132	0.0791	84.93532012	0.6532	0.0638

Data presented are the average values as percentages of the controls for the calculated growth velocity (CFU/mL/min) metric. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Values in bold denote statistically significant samples.

Table A7. Summary of growth curves challenging the Gram-negative pathogens with various phytochemicals at 0.5 mg/mL concentrations.

Gram-Negative Pathogens Growth Curve Average Endpoint States (%)						
Phytochemical (0.5 mg/mL)	<i>S. Typhimurium</i>			<i>P. aeruginosa</i>		
	Normalised Endpoint State (CFU/mL)	SEM (±)	<i>p</i> -Value	Normalised Endpoint State (CFU/mL)	SEM (±)	<i>p</i> -Value
Solvent control	100	1.07	-	100	0.06	-
Prosur	114.48	0.06	0.2964	88.45	1.04	0.2687
Eriodictyol	86.45	1.08	0.3283	101.77	0.08	0.8647
Ferulic acid	107.90	0.08	0.5678	99.85	0.08	0.9886
Quercetin	115.10	0.04	0.2762	101.78	0.03	0.8636
Caffeic acid	84.63	1.36	0.2679	98.32	0.06	0.8723
Thymol	33.22	0.76	<0.0001	70.53	0.12	0.0057
Kaempferol	113.03	0.10	0.3469	97.92	0.11	0.8417
Naringenin	53.76	1.44	0.0012	53.77	1.59	<0.0001
Vanillin	112.25	0.03	0.3764	87.32	1.02	0.2249

Data presented are the average values as percentages of the controls for the calculated endpoint state (CFU/mL) metric at the end of the 16 h incubation period. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Values in bold denote statistically significant samples.

Table A8. Summary of growth curves challenging the Gram-positive pathogens with various phytochemicals at 0.5 mg/mL concentrations.

Gram-Positive Pathogens Growth Curve Average Endpoint States (%)						
Phytochemical (0.5 mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
	Normalised Endpoint State (CFU/mL)	SEM (±)	<i>p</i> -Value	Normalised Endpoint State (CFU/mL)	SEM (±)	<i>p</i> -Value
Solvent control	100	0.05	-	100	0.12	-
Prosur	97.78	0.09	0.7720	61.21	1.35	<0.0001

Table A8. Cont.

Gram-Positive Pathogens Growth Curve Average Endpoint States (%)						
Phytochemical (0.5 mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
	Normalised Endpoint State (CFU/mL)	SEM (±)	<i>p</i> -Value	Normalised Endpoint State (CFU/mL)	SEM (±)	<i>p</i> -Value
Eriodictyol	55.89	0.18	<0.0001	64.69	0.26	<0.0001
Ferulic acid	97.96	0.06	0.7904	104.53	0.03	0.5754
Quercetin	85.21	0.95	0.0559	98.17	0.11	0.8206
Caffeic acid	73.99	0.84	0.0010	64.17	0.73	<0.0001
Thymol	56.81	0.27	<0.0001	29.84	0.81	<0.0001
Kaempferol	100.77	0.16	0.9194	102.77	0.05	0.7321
Naringenin	19.76	0.70	<0.0001	55.79	0.60	<0.0001
Vanillin	90.00	0.20	0.1934	102.92	0.03	0.7183

Data presented are the average values as percentages of the controls for the calculated endpoint state (CFU/mL) metric at the end of the 16 h incubation period. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Values in bold denote statistically significant samples.

Table A9. Summary of growth curves challenging the Gram-positive pathogens with various phytochemicals at 0.05 mg/mL concentrations.

Gram-Positive Pathogens Growth Curve Endpoint States (%)						
Phytochemical (0.05 mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
	Normalised Endpoint State (vCFU/mL)	SEM (±)	<i>p</i> -Value	Normalised Endpoint State (vCFU/mL)	SEM (±)	<i>p</i> -Value
Solvent control	100	0.22	-	100	0.12	-
Prosur	103.83	0.14	0.7205	105.73	0.03	0.4484
Eriodictyol	68.26	1.29	0.0038	99.64	0.14	0.9623
Ferulic acid	101.26	0.08	0.9069	104.34	0.07	0.5657
Quercetin	106.52	0.03	0.5427	54.06	1.17	<0.0001
Caffeic acid	107.36	0.03	0.4916	44.85	1.15	<0.0001
Thymol	25.86	1.15	<0.0001	99.63	0.06	0.9601
Kaempferol	102.30	0.21	0.8297	106.39	0.04	0.3983
Naringenin	22.61	0.80	<0.0001	102.74	0.03	0.7159
Vanillin	75.59	0.86	0.0246	84.58	0.56	0.0435

Data presented are the average values as percentages of the controls for the calculated endpoint state (CFU/mL) metric at the end of the 16 h incubation period. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Note the significant *p*-values obtained for the use of vanillin against *L. monocytogenes* at 0.05 mg/mL, which were not observed at 0.5 mg/mL. Values in bold denote statistically significant samples.

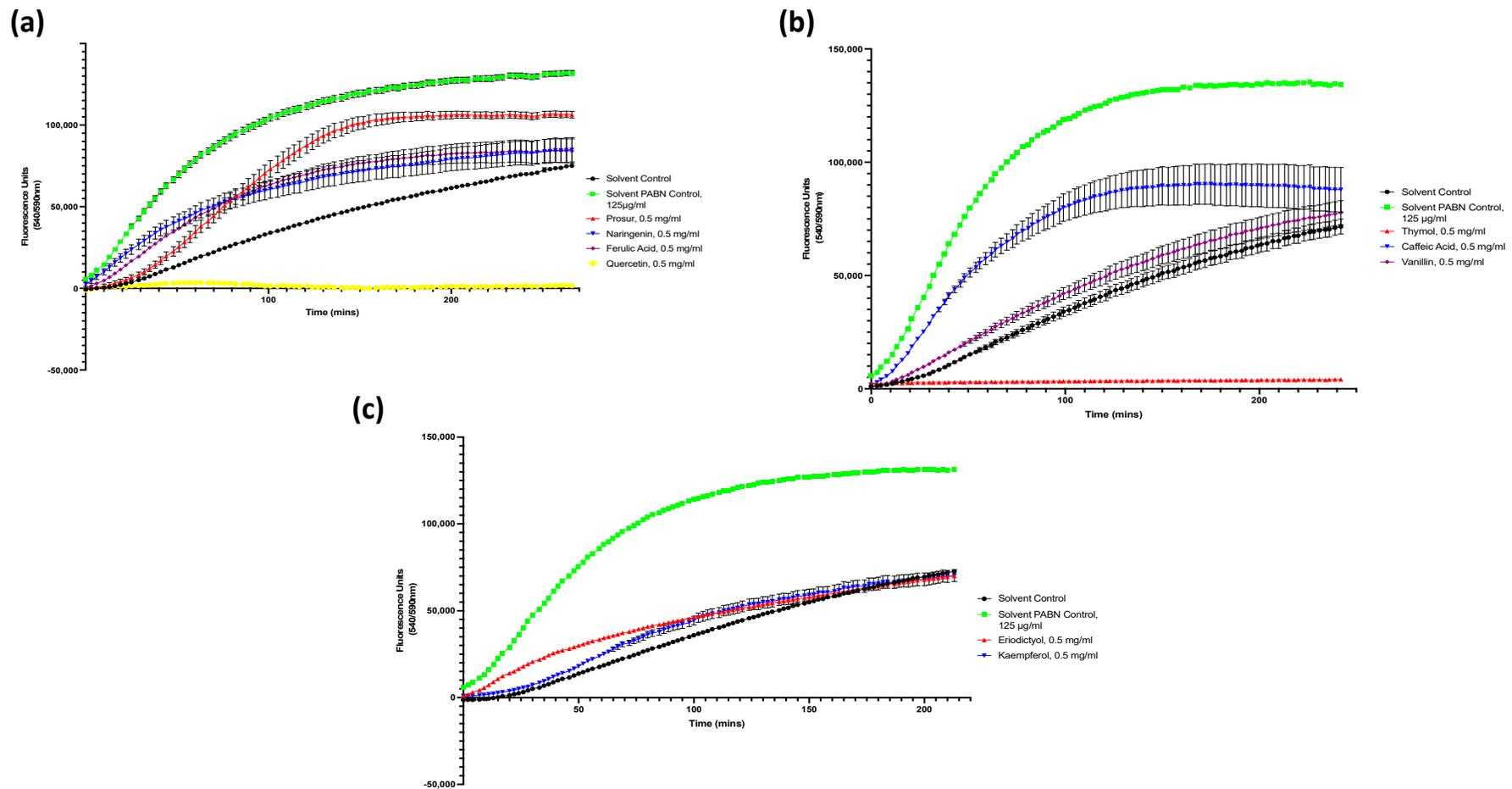


Figure A2. Drug accumulation curves of *S. Typhimurium* challenged with 0.5 mg/mL of all nine tested phytochemicals. (a) *S. Typhimurium* challenged with the Prosur NATPRE T-10+ mix, naringenin, ferulic acid, and quercetin. (b) *S. Typhimurium* challenged with thymol, caffeic acid, and vanillin. (c) *S. Typhimurium* challenged with eriodictyol and kaempferol. Points show the blank-adjusted and averaged values of five technical replicates for three biological replicates. Error bars indicate SEM (\pm).

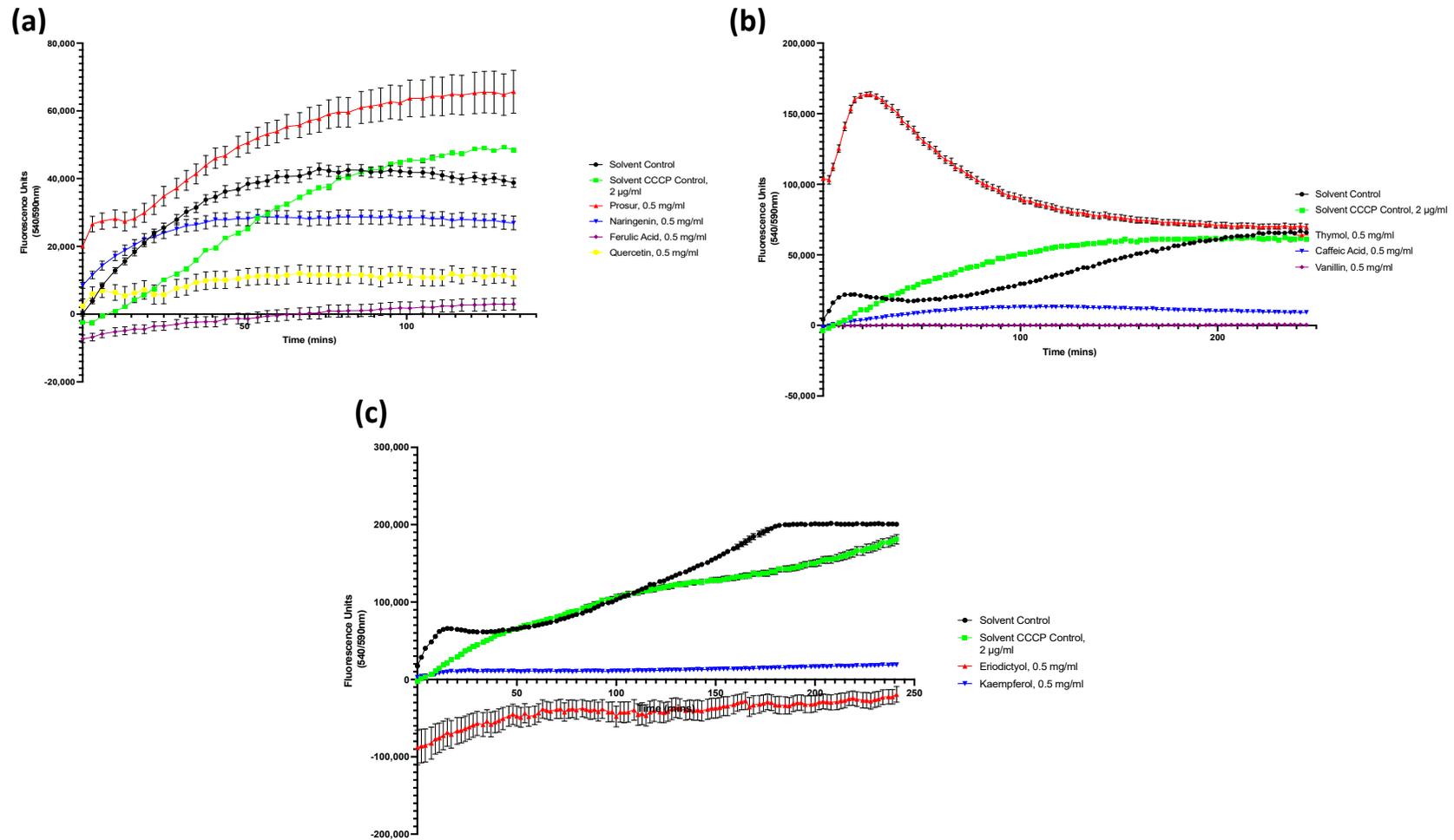


Figure A3. Drug accumulation curves of *S. aureus* challenged with 0.5 mg/mL of all nine tested phytochemicals. (a) *S. aureus* challenged with the Prosur NATPRE T-10+ mix, naringenin, ferulic acid, and quercetin. (b) *S. aureus* challenged with thymol, caffeic acid, and vanillin. (c) *S. aureus* challenged with eridictyol and kaempferol. Points show the blank-adjusted and averaged values of five technical replicates for three biological replicates. Error bars indicate SEM (\pm).

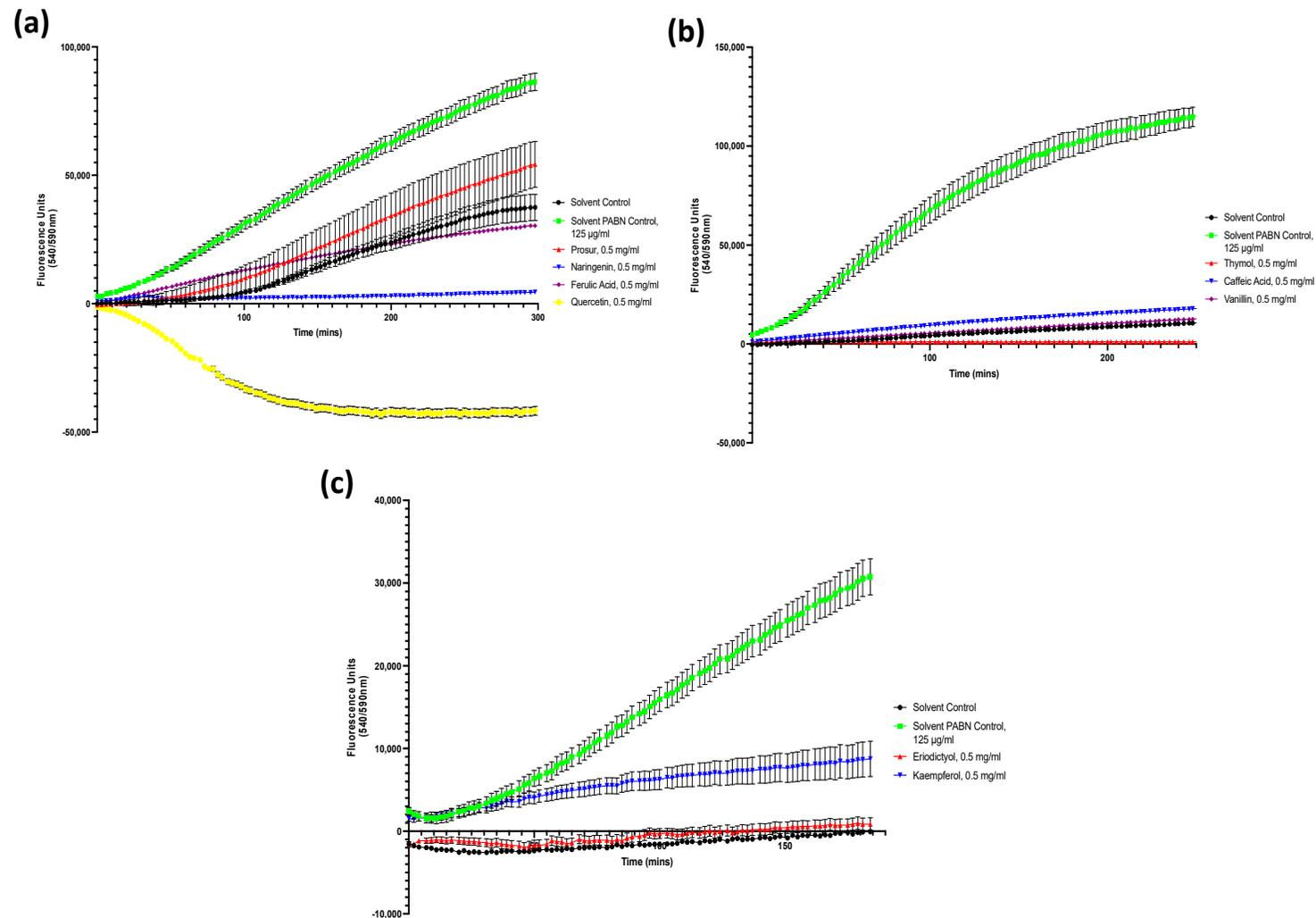


Figure A4. Drug accumulation curves of *P. aeruginosa* challenged with 0.5 mg/mL of all nine tested phytochemicals. (a) *P. aeruginosa* challenged with the Prosur NATPRE T-10+ mix, naringenin, ferulic acid, and quercetin. (b) *P. aeruginosa* challenged with thymol, caffeic acid, and vanillin. (c) *P. aeruginosa* challenged with eriodictyol and kaempferol. Points show the blank-adjusted and averaged values of five technical replicates for three biological replicates. Error bars indicate SEM (\pm).

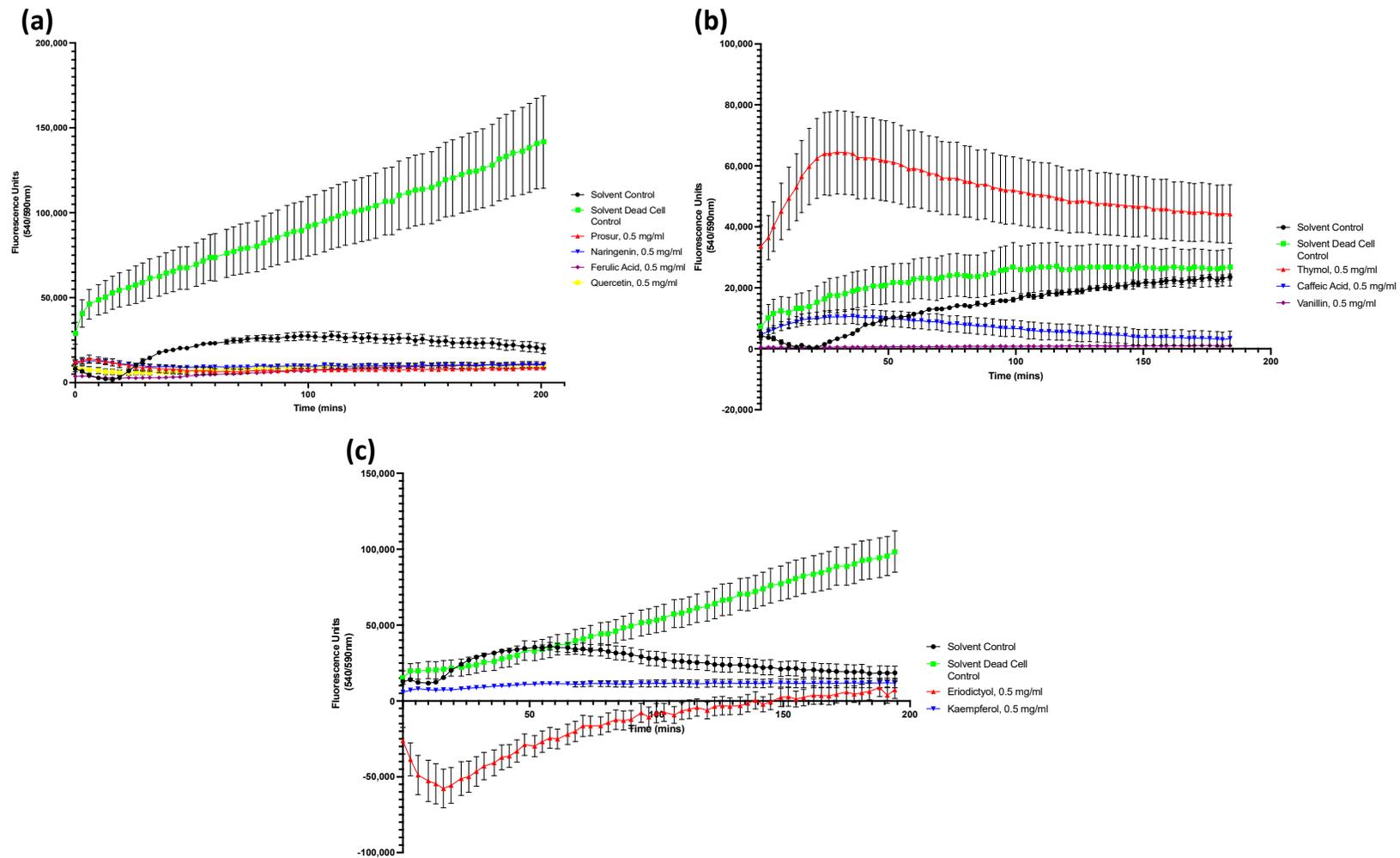


Figure A5. Drug accumulation curves of *L. monocytogenes* challenged with 0.5 mg/mL of all nine tested phytochemicals. (a) *L. monocytogenes* challenged with the Prosur NATPRE T-10+ mix, naringenin, ferulic acid, and quercetin. (b) *L. monocytogenes* challenged with thymol, caffeic acid, and vanillin. (c) *L. monocytogenes* challenged with eriodictyol and kaempferol. Points show the blank-adjusted and averaged values of five technical replicates for three biological replicates. Error bars indicate SEM (\pm).

Table A10. Summary of drug accumulation assays challenging the Gram-negative pathogens with various phytochemicals at 0.05 mg/mL concentrations.

Gram-Negative Pathogens Drug Accumulation Assays Average Fluorescence Accumulation Velocities (%)						
Phytochemical (0.5 mg/mL)	<i>S. Typhimurium</i>			<i>P. aeruginosa</i>		
	Normalised Fluorescence Accumulation Velocity (Fluorescence Units/min)	SEM (±)	<i>p</i> -Value	Normalised Fluorescence Accumulation Velocity (Fluorescence Units/min)	SEM (±)	<i>p</i> -Value
Solvent control	100	12.28	-	100	14.55	-
Positive control	197.30	5.95	<0.0001	506.62	30.74	<0.0001
Prosur	169.80	15.57	<0.0001	300.56	37.47	<0.0001
Eriodictyol	108.53	4.03	0.3430	24.96	2.73	0.0449
Ferulic acid	129.14	26.82	0.0014	186.53	3.56	0.0210
Quercetin	2.23	N/D	N/D	-282.52	N/D	N/D
Caffeic acid	140.80	47.14	<0.0001	108.54	3.41	0.8184
Thymol	2.36	0.43	<0.0001	4.18	0.21	0.0108
Kaempferol	108.91	18.82	0.3218	57.33	10.80	0.2521
Naringenin	117.93	30.66	0.0473	16.63	2.84	0.0261
Vanillin	104.80	25.57	0.5938	84.21	2.65	0.6713

Data presented are the average values as percentages of the controls for the calculated fluorescence accumulation velocity (fluorescence units/min) metric. Experiments were repeated with three biological replicates (five technical replicates each) over an incubation period of 16 h, trimmed to an appropriate timescale where the accumulation curves begun to plateau. Values in bold denote statistically significant samples.

Table A11. Summary of drug accumulation assays challenging the Gram-positive pathogens with various phytochemicals at 0.05 mg/mL concentrations.

Gram-Positive Pathogens Drug Accumulation Assays Average Fluorescence Accumulation Velocities (%)						
Phytochemical (0.5 mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
	Normalised Fluorescence Accumulation Velocity (Fluorescence Units/min)	SEM (±)	<i>p</i> -Value	Normalised Fluorescence Accumulation Velocity (Fluorescence Units/min)	SEM (±)	<i>p</i> -Value
Solvent control	100	97.15	-	100	12.02	-
Positive control	95.34	61.60	0.7157	565.91	64.85	<0.0001
Prosur	52.46	36.82	0.0003	-27.54	8.23	0.0310
Eriodictyol	62.45	80.66	0.0038	267.68	50.83	0.0048
Ferulic acid	12.96	5.768	<0.0001	57.69	2.36	0.4713
Quercetin	7.20	6.64	N/D	8.72	12.84	N/D
Caffeic acid	13.10	3.10	<0.0001	-10.70	9.43	0.0608
Thymol	-34.90	16.69	<0.0001	89.11	18.20	0.8528
Kaempferol	14.01	10.41	<0.0001	48.06	13.63	0.3768
Naringenin	16.69	9.03	<0.0001	-5.39	8.68	0.0740
Vanillin	1.38	1.48	<0.0001	5.02	0.55	0.1071

Data presented are the average values as percentages of the controls for the calculated fluorescence accumulation velocity (fluorescence units/min) metric. Experiments were repeated with three biological replicates (five technical replicates each) over an incubation period of 16 h, trimmed to an appropriate timescale where the accumulation curves began to plateau. Values in bold denote statistically significant samples.

Table A12. Summary of drug accumulation assays challenging the Gram-negative pathogens with various phytochemicals at 0.05 mg/mL concentrations.

Gram-Negative Pathogens Drug Accumulation Assays Average Steady State Fluorescence Accumulation (%)						
Phytochemical (0.5 mg/mL)	<i>S. Typhimurium</i>			<i>P. aeruginosa</i>		
	Normalised Steady State Fluorescence Accumulation (Fluorescence Units)	SEM (±)	<i>p</i> -Value	Normalised Steady State Fluorescence Accumulation (Fluorescence Units)	SEM (±)	<i>p</i> -Value
Solvent control	100	887.2	-	100	5517	-
Positive control	100	789.6	0.7690	370.21	4027	<0.0001
Prosur	76.99	9701	0.0007	318.34	3969	<0.0001
Eriodictyol	79.63	1133	0.0026	36.11	1192	<0.0001
Ferulic acid	59.64	11683	<0.0001	250.17	807.5	<0.0001
Quercetin	4.06	N/D	N/D	N/D	N/D	N/D
Caffeic acid	53.70	9890	<0.0001	107.66	1496	0.5869
Thymol	4.37	380.6	<0.0001	4.09	189.2	<0.0001
Kaempferol	51.63	1199	<0.0001	44.49	2722	<0.0001
Naringenin	88.52	2307	0.0867	74.02	1565	0.0668
Vanillin	75.64	6260	0.0003	171.61	3806	<0.0001

Data presented are the average values as percentages of the controls for the calculated steady state fluorescence accumulation (fluorescence units/min) metric. Experiments were repeated with three biological replicates (five technical replicates each) over an incubation period of 16 h, trimmed to an appropriate timescale where the accumulation curves began to plateau. Values in bold denote statistically significant samples.

Table A13. Summary of drug accumulation assays challenging the Gram-positive pathogens with various phytochemicals at 0.05 mg/mL concentrations.

Gram-Positive Pathogens Drug Accumulation Assays Average Steady State Fluorescence Accumulation (%)						
Polyphenol (0.5 mg/mL)	<i>S. aureus</i>			<i>L. monocytogenes</i>		
	Normalised Steady State Fluorescence Accumulation (Fluorescence Units)	SEM (±)	<i>p</i> -Value	Normalised Steady State Fluorescence Accumulation (Fluorescence Units)	SEM (±)	<i>p</i> -Value
Solvent control	100	20488	-	100	2901	-
Positive control	108.03	20032	0.5893	1024.55	23660	<0.0001
Prosur	106.33	9631	0.6704	809.83	15129	<0.0001
Eriodictyol	16.82	11308	<0.0001	128.89	2630	0.7475
Ferulic acid	13.11	1894	<0.0001	71.58	769.6	0.7515
Quercetin	5.91	N/D	N/D	12.27	1095	N/D
Caffeic acid	7.02	986.9	<0.0001	-1.51	1590	0.2588
Thymol	93.37	3219	0.6558	386.07	10075	0.0017
Kaempferol	31.46	3307	<0.0001	148.95	1307	0.5855
Naringenin	20.46	3114	<0.0001	59.46	1381	0.6515
Vanillin	0.77	155.5	<0.0001	11.02	330.2	0.3220

Data presented are the average values as percentages of the controls for the calculated steady-state fluorescence accumulation (fluorescence units/min) metric. Experiments were repeated with three biological replicates (five technical replicates each) over an incubation period of 16 h, trimmed to an appropriate timescale where the accumulation curves began to plateau. Values in bold denote statistically significant samples.

Viable numbers (based on CFU/mL) of the *S. Typhimurium* parental and two thymol-selected mutant strains. Experiments were repeated with three biological replicates (three technical replicates each) over an incubation period of 16 h. Graphs display the averaged values of three technical replicates for three biological replicates. Error bars indicate SEM (±).

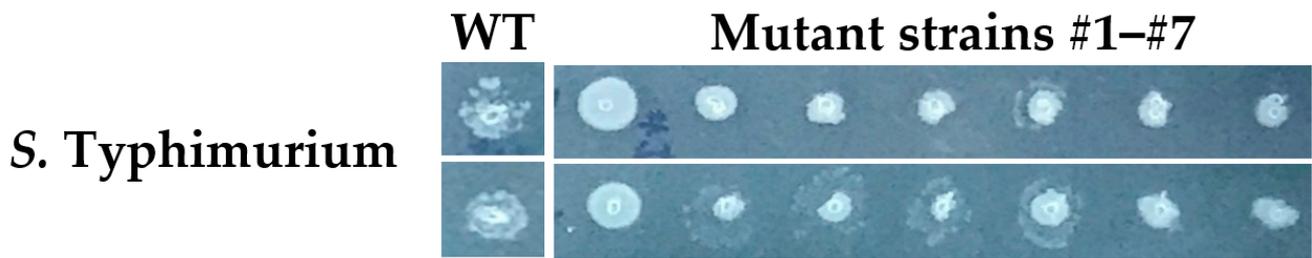


Figure A6. Colony morphologies of thymol-selected mutant and parental strains of *S. Typhimurium*. *S. Typhimurium* parental and thymol-selected mutant colonies grown overnight at 37 °C on LB agar laced with 0.25 mg/mL thymol. Note the larger, healthier colonies of mutant strain #1, in particular. The experiment was performed in biological duplicate.

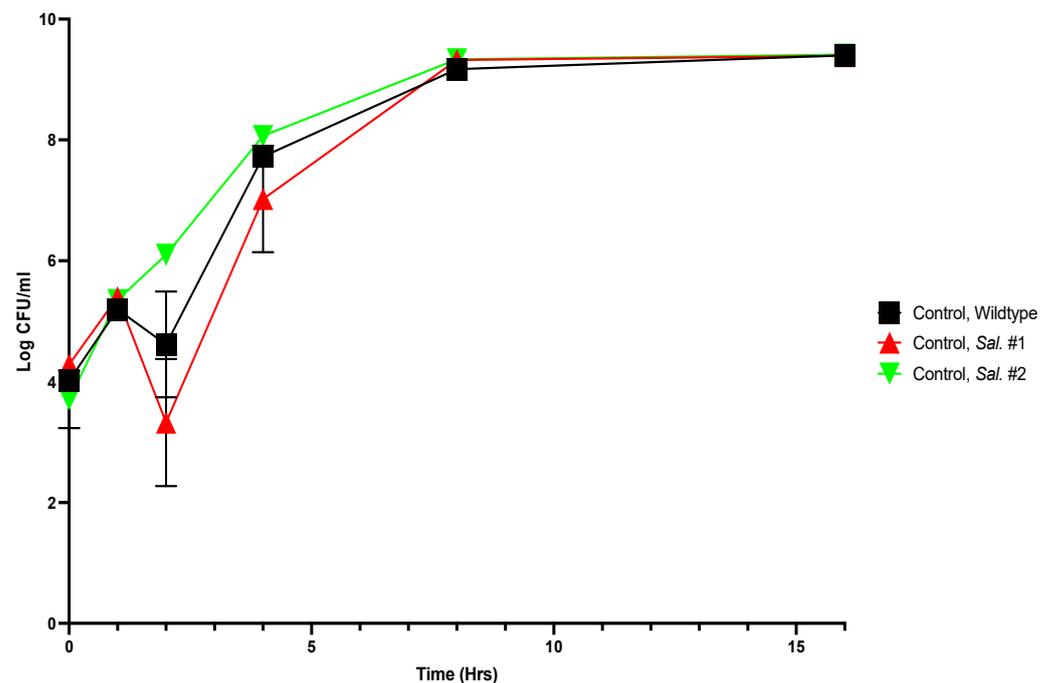


Figure A7. Growth curves of thymol-selected *S. Typhimurium* mutants, unchallenged by thymol.

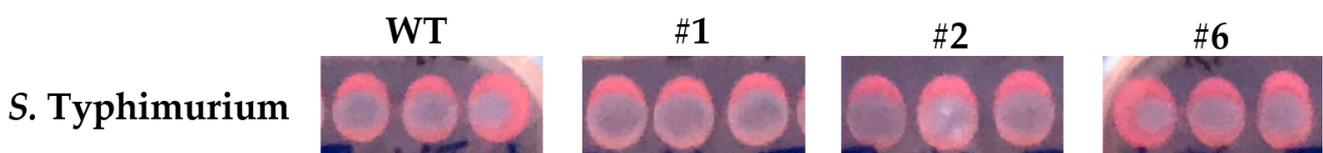


Figure A8. Congo red staining of the *S. Typhimurium* parental and thymol-selected mutant colonies. *S. Typhimurium* parental and thymol-selected mutant colonies grown overnight at 37 °C on LB-NaCl agar laced with 40 µg/mL congo red. Note the lesser degree of congo red staining for the *S. Typhimurium* mutant strains #1 and #2 compared to the parental strain. Compare these also to mutant strain #6, which lacks efflux-associated SNPs. The experiment was performed in biological duplicate.

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