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Mechanizm antybiotycznego działania aldehydu *trans*-
cynamonowego na szczepy *Escherichia coli*

Mechanism of antibacterial action of *trans*-
cinnamaldehyde against *Escherichia coli*

Praca przedstawiona
Radzie Dyscypliny Nauki biologiczne Uniwersytetu Gdańskiego
celem uzyskania stopnia doktora
w dziedzinie nauk ścisłych i przyrodniczych
w dyscyplinie nauki biologiczne

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GDAŃSK 2026

Podziękowania

Serdecznie dziękuję mojej Promotor, prof. dr hab. Agnieszce Szalewskiej-Pałasz, za opiekę naukową, cenne wskazówki, dzielenie się wiedzą oraz wsparcie podczas realizacji badań i przygotowywania rozprawy.

Szczególne podziękowania kieruję do mojego Promotora Pomocniczego, dr Dariusza Nowickiego. Dziękuję za współpracę na każdym etapie realizacji tej pracy, za poświęcony czas, liczne dyskusje nad wynikami i kierunkiem badań oraz za gotowość do pomocy zawsze, gdy jej potrzebowałam. Bez tego zaangażowania powstanie tej rozprawy byłoby znacznie trudniejsze.

dr Monice Maciąg-Dorszyńskiej dziękuję za współpracę, wzajemną pomoc, dzielenie się doświadczeniem oraz codzienną życzliwość. Dziękuję za wszystkie rozmowy, wymianę pomysłów i wspólnie spędzony czas w laboratorium.

Dziękuję również wszystkim osobom, które przyczyniły się do powstania tej rozprawy.

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1. WYKAZ PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Praca oryginalna:

Antibacterial activity of *t*-cinnamaldehyde: an approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)

Karczewska Monika, Wang Ai Yan, Narajczyk Magdalena, Słomiński Bartosz, Szalewska-Pałasz Agnieszka, Nowicki Dariusz. *Phytomedicine : international journal of phytotherapy and phytopharmacology* 2024, 132:155845.

DOI:10.1016/j.phymed.2024.155845

IF₂₀₂₄: 8.3; MNiSW: 140

Praca oryginalna:

Increased levels of (p)ppGpp correlate with virulence and biofilm formation, but not with growth, in strains of uropathogenic *Escherichia coli*

Karczewska Monika, Strzelecki Patryk, Bogucka Krystyna, Potrykus Katarzyna, Szalewska-Pałasz Agnieszka, Nowicki Dariusz. *International Journal of Molecular Sciences* 2023, 24(4). DOI:10.3390/ijms24043315

IF₂₀₂₃: 5,6; MNiSW: 140

Praca oryginalna:

Trans*-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic *Escherichia coli

Karczewska Monika, Strzelecki Patryk, Pyrczak-Felczykowska Agnieszka, Zima Katarzyna, Szalewska-Pałasz Agnieszka, Nowicki Dariusz. *Biomedicine & pharmacotherapy* 2026, 198:119306. DOI:10.1016/j.biopha.2026.119306

IF₂₀₂₆: 7,5; MNiSW: 100

Nierecenzowany manuskrypt pracy oryginalnej:

Spicing up urinary tract infections: synergistic action of fosfomycin with *trans*-cinnamaldehyde - insight to the mode of action

Karczewska Monika, Strzelecki Patryk, Maciąg-Dorszyńska Monika, Kapusta Małgorzata, Pyrczak-Felczykowska Agnieszka, Szalewska-Pałasz Agnieszka, Nowicki Dariusz. Praca zdeponowana w repozytorium bioRxiv jako nierecenzowany manuskrypt, 2026

DOI: 10.64898/2026.06.23.733984

Praca przeglądowa:

Phytochemicals controlling enterohemorrhagic *Escherichia coli* (EHEC) virulence - current knowledge of their mechanisms of action

Strzelecki Patryk, Karczewska Monika, Szalewska-Pałasz Agnieszka, Nowicki Dariusz. *International Journal of Molecular Sciences* 2025, 26(1).

DOI:10.3390/ijms26010381

IF₂₀₂₅: 5,6; MNiSW: 140

Łączna wartość wskaźnika oddziaływania (IF): 27

Łączna punktacja MNiSW: 520

Prace naukowe wchodzące w skład rozprawy doktorskiej były finansowane z grantu Narodowego Centrum Nauki (NCN) numer UMO-2018/31/D/NZ7/02258 przyznanego dr Dariuszowi Nowickiemu.



2. WYKAZ SKRÓTÓW:

SKRÓT	ZNACZENIE
agn43	gen kodujący antygen 43
aer	gen kodujący aerobaktynę
CadA	dekarboksylaza lizynowa
cnfl	gen kodujący cytotoksyczny czynnik martwicy 1
EFSA	Europejski Urząd ds. Bezpieczeństwa Żywności (<i>European Food Safety Authority</i>)
EHEC	enterokrwotoczne szczepy <i>Escherichia coli</i>
ExPEC	zewnątrzjelitowe patogenne szczepy <i>Escherichia coli</i>
FDA	Amerykańska Agencja Żywności i Leków (<i>Food and Drug Administration</i>)
fimH	gen kodujący adhezynę fimbrialną typu 1
FOS	fosfomycyna
GFP	białko zielonej fluorescencji (<i>green fluorescent protein</i>)
GO	ontologia genów
HB2	linia ludzkich komórek nabłonka piersi
HDFa	ludzkie fibroblasty skórne (<i>human dermal fibroblasts, adult</i>)
hlyA	gen kodujący α -hemolizynę
HUS	zespół hemolityczno-mocznicowy
ibeA	gen odpowiedzialny za inwazję komórek śródbłonki mózgu A
IL-1 β	interleukina 1 β
IL-6	interleukina 6
IL-10	interleukina 10
iss	gen warunkujący zwiększone przeżycie w surowicy
KEGG	encyklopedia genów i genomów Kioto (<i>Kyoto Encyclopedia of Genes and Genomes</i>)
lexA	gen kodujący represor odpowiedzi SOS
MBC	minimalne stężenie bakteriobójcze (<i>minimum bactericidal concentration</i>)
MIC	minimalne stężenie hamujące (<i>minimum inhibitory concentration</i>)
MIC ₉₀	stężenie hamujące wzrost 90% badanych szczepów
NAD ⁺ /NADH	dinukleotyd nikotynoamidoadeninowy (forma utleniona/zredukowana)
nar	operon reduktazy azotanowej
papC	gen fimbrii P
PBMC	jednojądrzaste komórki krwi obwodowej (<i>peripheral blood mononuclear cells</i>)
poxB	gen kodujący oksydazę pirogronianową
recA	gen kodujący białko RecA uczestniczące w odpowiedzi SOS
RelA	syntetaza alarmonów odpowiedzi ścisłej
RNA-seq	sekwencjonowanie transkryptomu
ROS	reaktywne formy tlenu
sfa	gen kodujący adhezynę S-fimbrialną
sitA	gen kodujący transporter żelaza SitA
SOS	bakteryjna odpowiedź SOS
spoT	gen kodujący syntetazę/hydrolazę alarmonów odpowiedzi ścisłej
stx	gen kodujący toksynę Shiga
sulA	gen kodujący inhibitor podziału komórkowego odpowiedzi SOS
t-CA	aldehid <i>trans</i> -cynamonowy
TCA	cykl kwasu trikarboksylowego (<i>tricarboxylic acid cycle</i>)
TNF- α	czynnik martwicy nowotworów α (<i>tumor necrosis factor alpha</i>)
tor	operon reduktazy trimetyloaminy N-tlenku

UPEC uropatogenne szczepy *Escherichia coli*
UTI zakażenia układu moczowego
ZUM zakażenia układu moczowego
(p)ppGpp guanozyno-tetra- i pentafosforan, alarmony odpowiedzi ścisłej

3. SŁOWA KLUCZOWE

aldehyd *trans* cynamonowy, *Escherichia coli*, UPEC, EHEC, toksyna Shiga, odpowiedź ścisła, ppGpp, wirulencja, biofilm, *Galleria mellonella*, antybiotykooporność

trans-cinnamaldehyde, *Escherichia coli*, UPEC, EHEC, Shiga toxin, stringent response, ppGpp, virulence, biofilm, *Galleria mellonella*, antibiotic resistance

4. STRESZCZENIE W JĘZYKU POLSKIM

Niniejsza rozprawa doktorska została przygotowana w formie cyklu trzech publikacji oryginalnych, jednego artykułu opublikowanego w formie preprintu i jednej publikacji przeglądowej. Rozprawa dotyczy przeciwdrobnoustrojowego oraz antywirulencyjnego mechanizmu działania aldehydu *trans*-cynamonowego (*t*-CA) wobec patogennych szczepów *Escherichia coli*. Badania przeprowadzono z wykorzystaniem szczepów należących do dwóch istotnych klinicznie patotypów – uropatogennych (*uropathogenic E. coli*, UPEC) oraz enterokrwotocznych (*enterohemorrhagic E. coli*, EHEC) – odpowiedzialnych odpowiednio za zakażenia układu moczowego oraz ciężkie zakażenia przewodu pokarmowego.

Głównym celem pracy było poznanie mechanizmu działania aldehydu *trans*-cynamonowego wobec patogennych szczepów *E. coli*. W szczególności analizowano wpływ badanego związku na przeżywalność bakterii, regulację procesów związanych z wirulencją i tworzeniem biofilmu oraz jego udział w indukcji odpowiedzi ścisłej. Dodatkowo oceniono potencjał aldehydu jako czynnika wspomagającego działanie antybiotyków oraz jego aktywność w modelach *in vitro* i *in vivo*.

Uzyskane wyniki wykazały, że *t*-CA charakteryzuje się szeroką aktywnością przeciwdrobnoustrojową wobec szczepów *E. coli*, niezależnie od ich profilu wirulencji. Działanie związku obejmowało zaburzenie integralności błony komórkowej, zahamowanie procesów biosyntetycznych oraz głęboką reorganizację metabolizmu komórkowego. Wykazano, że ekspozycja bakterii na *t*-CA prowadzi do indukcji odpowiedzi ścisłej zależnej od RelA, której aktywacja wiąże się z przeprogramowaniem ekspresji genów i przejściem komórki w stan adaptacyjny charakterystyczny dla warunków stresowych.

Szczególnie istotnym aspektem pracy było wykazanie wpływu *t*-CA na ograniczenie wirulencji szczepów EHEC. Związek hamował rozwój bakteriofagów kodujących toksynę Shiga oraz obniżał ekspresję genów *stx* zatem może ograniczać patogenność bakterii bez ryzyka nasilania przebiegu zakażenia.

W pracy wykazano ponadto, że *t*-CA skutecznie zaburza proces tworzenia biofilmu przez szczepy UPEC, prowadząc do zmniejszenia biomasy biofilmu oraz ograniczenia syntezy składników macierzy pozakomórkowej. Analiza transkryptomyczna RNA-seq potwierdziła, że działanie *t*-CA obejmuje globalne zmiany w ekspresji genów związanych z metabolizmem energetycznym, odpowiedzią na stres, ruchem komórkowym oraz procesami regulującymi kolonizację i wirulencję.

Ważnym elementem badań była ocena możliwości zastosowania *t*-CA w terapii skojarzonej z fosfomicyną, gdzie wykazano synergistyczne działanie obu związków zarówno wobec wolno żyjących komórek, jak i biofilmu bakteryjnego. Połączenie *t*-CA z fosfomicyną zwiększało skuteczność przeciwdrobnoustrojową oraz ograniczało rozwój oporności podczas długotrwałej ekspozycji bakterii na antybiotyki. Jednocześnie badania przeprowadzone w modelu *Galleria mellonella* potwierdziły skuteczność ochronną terapii skojarzonej *in vivo*. Uzyskane wyniki wskazują, że aldehyd *trans*-cynamonowy oddziałuje wielokierunkowo na komórkę bakteryjną, łącząc działanie przeciwdrobnoustrojowe z modulacją procesów odpowiedzialnych za wirulencję i adaptację do stresu. Ważną rolę w mechanizmie jego działania odgrywa odpowiedź ścisła, integrująca zmiany metaboliczne i regulacyjne zachodzące w komórce. Przedstawione dane sugerują, że *t*-CA może stanowić obiecujący czynnik wspomagający rozwój nowych strategii antywirulencyjnych i terapii skojarzonych wobec patogennych szczepów *E.coli*.

5. STRESZCZENIE W JĘZYKU ANGIELSKIM

This doctoral dissertation was prepared as a series of three peer-reviewed original publications, one preprint article, and one review article. The dissertation focuses on the antimicrobial and antivirulence mechanisms of action of trans-cinnamaldehyde (*t*-CA) against pathogenic *Escherichia coli* strains. The study was conducted using strains representing two clinically significant pathotypes—uropathogenic *E. coli* (UPEC) and enterohemorrhagic *E. coli* (EHEC)—which are responsible for urinary tract infections and severe gastrointestinal diseases, respectively.

The primary objective of this dissertation was to elucidate the mechanism of action of trans-cinnamaldehyde against pathogenic *E. coli* strains. Particular emphasis was placed on evaluating the effects of *t*-CA on bacterial survival, the regulation of virulence- and biofilm-related processes, and its role in the induction of the stringent response. Additionally, the potential of *t*-CA as an adjuvant enhancing antibiotic activity was assessed, together with its efficacy in both *in vitro* and *in vivo* models.

The obtained results demonstrated that *t*-CA exhibits broad-spectrum antimicrobial activity against *E. coli* strains irrespective of their virulence profiles. Its mode of action involves disruption of bacterial cell membrane integrity, inhibition of essential biosynthetic processes, and extensive reprogramming of cellular metabolism. Exposure of bacterial cells to *t*-CA was shown to induce a RelA-dependent stringent response, the activation of which is associated with global changes in gene expression and the transition of cells into an adaptive physiological state characteristic of stress conditions.

A particularly significant finding of this work was the demonstration of the antivirulence activity of *t*-CA against EHEC strains. The compound inhibited the development of Shiga toxin-encoding bacteriophages and reduced the expression of *stx* genes, suggesting its potential to attenuate bacterial pathogenicity without increasing the severity of infection.

Furthermore, *t*-CA was found to effectively impair biofilm formation by UPEC strains, leading to a reduction in biofilm biomass and decreased synthesis of extracellular matrix components. RNA-sequencing (RNA-seq) transcriptomic analysis confirmed that the activity of *t*-CA is associated with global alterations in the expression of genes involved in energy metabolism, stress response, cellular motility, and regulatory pathways governing colonization and virulence.

An additional important aspect of this research was the evaluation of *t*-CA in combination therapy with fosfomycin. A synergistic interaction between the two compounds was

demonstrated against both planktonic bacterial cells and established biofilms. The combination of *t*-CA and fosfomycin enhanced antimicrobial efficacy and limited the emergence of antibiotic resistance during prolonged bacterial exposure to fosfomycin. Moreover, experiments conducted using the *G. mellonella* infection model confirmed the protective efficacy of the combined treatment *in vivo*.

Collectively, the findings indicate that trans-cinnamaldehyde exerts pleiotropic effects on bacterial cells by combining direct antimicrobial activity with modulation of virulence-associated and stress-adaptive processes. The stringent response appears to play a central role in its mechanism of action, integrating the metabolic and regulatory changes induced within the bacterial cell. These results suggest that *t*-CA represents a promising candidate for the development of novel antivirulence strategies and combination therapies targeting pathogenic *E. coli* strains.

6. WPROWADZENIE

Escherichia coli jest Gram-ujemną bakterią należącą do naturalnej mikrobioty przewodu pokarmowego człowieka i zwierząt. Stanowi jeden z najważniejszych drobnoustrojów z punktu widzenia medycyny i weterynarii [1, 2]. Część szczepów wykształciła mechanizmy umożliwiające wywoływanie zakażeń jelitowych oraz pozajelitowych [1, 2]. Populacje występujące u zwierząt gospodarskich mogą stanowić rezerwuuar szczepów o potencjale zoonotycznym, a także genów związanych z opornością na środki przeciwdrobnoustrojowe i wirulencją, które mogą ujawniać się w zakażeniach u ludzi [2]. W badaniach stojących u podstawy tej pracy szczególne znaczenie przypisuje się dwóm patotypom: uropatogennym szczepom *E. coli* (UPEC) oraz enterokrwotocznym szczepom *E. coli* (EHEC) [3, 4].

Zakażenia układu moczowego (UTI, *urinary tract infections*) należą do najczęściej występujących chorób bakteryjnych na świecie i stanowią istotny problem zdrowia publicznego [5, 6]. Szacuje się, że rocznie dochodzi do około 150 milionów przypadków UTI, co generuje znaczne koszty diagnostyki i leczenia [7]. UPEC odpowiadają za około 80% niepowikłanych zakażeń układu moczowego oraz znaczną część zakażeń szpitalnych [8, 9]. Szczepy te należą do grupy ExPEC (*extraintestinal pathogenic E. coli*) i posiadają liczne czynniki wirulencji umożliwiające skuteczną kolonizację dróg moczowych [10]. Kluczową rolę odgrywają adhezyny, w tym fimbrie typu 1 oraz fimbrie P, warunkujące przyleganie bakterii do komórek nabłonka. Istotne znaczenie mają również toksyny, takie jak hemolizyna α oraz CNF1, a także systemy pozyskiwania żelaza umożliwiające przeżycie bakterii w środowisku ubogim w ten pierwiastek [10, 11].

Jednym z najważniejszych elementów patogenezы UPEC jest zdolność do tworzenia biofilmu [12]. Biofilm stanowi wielokomórkową strukturę bakterii otoczoną macierzą zewnątrzkomórkową, która zwiększa przeżywalność drobnoustrojów w niekorzystnych warunkach środowiskowych oraz ogranicza skuteczność odpowiedzi immunologicznej i antybiotykoterapii [13]. Struktura biofilmu chroni bakterie przed działaniem sił hydrodynamicznych oraz mechanizmów obronnych gospodarza. W konsekwencji szczepy zdolne do tworzenia biofilmu wykazują zwiększoną tolerancję na działanie antybiotyków oraz większą zdolność do wywoływania zakażeń przewlekłych i nawrotowych [12, 14]. Narastająca antybiotykooporność szczepów UPEC stanowi obecnie jedno z największych wyzwań współczesnej medycyny [15]. Powszechne stosowanie antybiotyków prowadzi

do selekcji szczepów wielolekoopornych wykazujących oporność między innymi na fluorochinolony, β -laktamy oraz cefalosporyny [6]. Problem ten jest szczególnie istotny w przypadku zakażeń nawrotowych wymagających wielokrotnej antybiotykoterapii. W odpowiedzi na ograniczającą się skuteczność klasycznych terapii ponownie wzrosło zainteresowanie fosfomycyną (FOS) – antybiotykiem hamującym pierwszy etap biosyntezy peptydoglikanu ściany komórkowej bakterii [16]. Dzięki unikalnemu mechanizmowi działania oraz korzystnym właściwościom farmakokinetycznym pozostaje jednym z podstawowych antybiotyków stosowanych w terapii niepowikłanych zakażeń dolnych dróg moczowych. Antybiotyk ten zachowuje wysoką skuteczność również wobec wielu szczepów wielolekoopornych [17]. Jednocześnie obserwowane są przypadki obniżonej wrażliwości bakterii na fosfomycynę, co wskazuje na potrzebę dalszych badań nad strategiami pozwalającymi utrzymać lub zwiększyć skuteczność tego leku [16, 18].

Drugim istotnym patotypem *E. coli* są enterokrwotoczne szczepy EHEC, które odpowiadają za ciężkie zakażenia przewodu pokarmowego przebiegające z krwotocznym zapaleniem jelita grubego, a w najcięższych przypadkach prowadzące do rozwoju zespołu hemolityczno-mocznicowego (HUS, *hemolytic uremic syndrome*) związanego z niedokrwistością hemolityczną, ostrą niewydolnością nerek i trombocytopenią. Głównym czynnikiem wirulencji EHEC jest toksyna Shiga kodowana przez geny obecne w genomach bakteriofagów lambdoidalnych zintegrowanych z chromosomem bakteryjnym [19-21]. Ekspresja toksyn Shiga związana jest bezpośrednio z aktywacją cyklu litycznego bakteriofagów, co prowadzi do uwalniania toksyn podczas lizy komórki bakteryjnej [22]. Z tego względu leczenie zakażeń EHEC pozostaje szczególnie problematyczne, ponieważ wiele antybiotyków indukuje odpowiedź SOS bakterii, aktywację profagów oraz wzrost produkcji toksyn Shiga (Stx) [23]. W konsekwencji klasyczna antybiotykoterapia może zwiększać ryzyko rozwoju HUS, szczególnie u dzieci i osób starszych [20]. Ograniczone możliwości terapeutyczne sprawiają, że poszukiwanie nowych strategii przeciwdrobnoustrojowych oraz przeciwwirulencyjnych stanowi jeden z najważniejszych kierunków współczesnych badań nad leczeniem zakażeń układu moczowego.

W kontekście obu opisanych problemów klinicznych rośnie zainteresowanie strategiami alternatywnymi wobec klasycznej antybiotykoterapii. Globalne narastanie oporności bakterii osłabia skuteczność dostępnych metod leczenia, co prowadzi do wzrostu

zainteresowania związkami pochodzenia naturalnego. Szczególną uwagę zwrócono na fitozwiązki oraz składniki olejków eterycznych, które mogą wpływać na metabolizm bakterii, tworzenie biofilmu, zmysł gęstościowy (quorum sensing) oraz ekspresję czynników wirulencji [24-26]. W przeciwieństwie do klasycznych antybiotyków wiele naturalnych związków nie działa wyłącznie bakteriobójczo, lecz ogranicza zdolność bakterii do kolonizacji organizmu gospodarza oraz wywoływania zakażeń. Takie podejście może potencjalnie ograniczać presję selekcyjną odpowiedzialną za rozwój oporności bakteryjnej [27]. Jednym z fitozwiązków o właściwościach przeciwdrobnoustrojowych jest aldehyd *trans*-cynamonowy (*t*-CA), będący głównym składnikiem olejku z kory cynamonu [28]. Związek ten wykazuje szerokie spektrum aktywności biologicznej obejmujące działanie przeciwbakteryjne czy przeciwgrzybicze. Mimo że od dawna wykorzystywany jest do zwalczania różnych zakażeń, molekularne podstawy jego działania nadal nie zostały w pełni wyjaśnione [29]. Dotychczasowe badania wskazują, że związek ten może wpływać na różne aspekty funkcjonowania komórki bakteryjnej, w tym na integralność błony komórkowej, metabolizm oraz procesy związane z wirulencją i tworzeniem biofilmu [26, 29]. Pomimo licznych doniesień dotyczących jego aktywności nadal nie jest jasne, czy działanie tego związku ogranicza się do nieswoistego uszkodzenia komórki bakteryjnej, czy obejmuje również bardziej złożone zmiany w funkcjonowaniu procesów regulacyjnych bakterii. W związku z tym zasadne jest dalsze badanie wpływu tego typu związków na fenotyp patogenny bakterii, nie tylko w kontekście ich działania przeciwdrobnoustrojowego i antywirulencyjnego, lecz także potencjalnego wykorzystania jako substancji wspomagających skuteczność terapii antybiotykowej. Tym bardziej, że związek uznany jest przez EFSA i FDA za bezpieczny oraz wykazujący niską toksyczność. Aldehyd *trans*-cynamonowy postrzegany jest więc jako obiecujący kandydat do zastosowań terapeutycznych [30].

Wspólną cechą obu opisanych patotypów jest fakt, że ich patogenność nie wynika wyłącznie z obecności pojedynczych genów wirulencji, lecz także ze zdolności do koordynacji procesów metabolicznych, adaptacyjnych i regulacyjnych umożliwiających przeżycie w organizmie gospodarza oraz utrzymanie zakażenia. Patogenność bakterii stanowi zatem efekt działania złożonych sieci regulacyjnych łączących odbiór sygnałów środowiskowych z odpowiednią modulacją ekspresji genów. Skuteczna kolonizacja organizmu gospodarza wymaga dynamicznego dostosowywania procesów fizjologicznych obejmujących wzrost, gospodarowanie energią, ekspresję czynników adhezyjnych,

tworzenie biofilmu, pozyskiwanie składników odżywczych oraz aktywację mechanizmów odpowiedzi na stres.

Jednym z najważniejszych układów integrujących te procesy jest odpowiedź ścisła (*stringent response*) [31, 32]. Jej głównymi efektorami są nukleotydowe alarmony ppGpp i pppGpp, określane wspólnie jako (p)ppGpp [33]. W bakteriach Gram-ujemnych, w tym w *E. coli*, metabolizm alarmonów (p)ppGpp regulowany jest głównie przez enzymy RelA oraz SpoT [31, 32]. RelA odpowiada przede wszystkim za syntezę alarmonów, natomiast SpoT uczestniczy zarówno w ich syntezie, jak i degradacji. Odpowiedź ścisła była tradycyjnie opisywana jako mechanizm aktywowany w warunkach niedoboru aminokwasów lub innych czynników stresowych, prowadzący do ograniczenia procesów wzrostowych i przejścia komórki w stan sprzyjający przetrwaniu [32]. Współczesne badania wskazują jednak, że rola (p)ppGpp wykracza poza klasyczną odpowiedź na stres. Alarmony te pełnią funkcję globalnych regulatorów wpływających na ekspresję genów związanych z metabolizmem, adaptacją oraz odpowiedzią na stres środowiskowy [31, 34]. Coraz wyraźniej podkreśla się również ich znaczenie w regulacji procesów bezpośrednio związanych z patogenizacją, takich jak ekspresja czynników wirulencji, tworzenie biofilmu, kolonizacja nisz gospodarza oraz przetrwanie w warunkach ograniczonej dostępności składników odżywczych [34]. W przypadku enterokrwotocznych szczepów *E. coli* wykazano ponadto, że odpowiedź ścisła może wpływać na regulację cyklu bakteriofagów kodujących toksynę Shiga, a tym samym modulować poziom ekspresji toksyn Stx [35]. Tym samym (p)ppGpp należy postrzegać nie tylko jako regulatory odpowiedzi na stres, lecz także jako element nadrzędnej sieci regulacyjnej integrującej metabolizm, adaptację i potencjał chorobotwórczy komórki bakteryjnej.

7. CEL PRACY

Celem badań było określenie aktywności przeciwdrobnoustrojowej aldehydu *trans*-cynamonowego wobec szczepów *Escherichia coli* oraz wyjaśnienie molekularnych mechanizmów jego działania.

Cele szczegółowe:

1. Charakterystyka klinicznych szczepów uropatogennych *E.coli* pod względem cech związanych z wirulencją i adaptacją do środowiska gospodarza.
2. Ocena zależności pomiędzy tworzeniem biofilmu, odpowiedzią ścisłą oraz wirulencją szczepów UPEC.
3. Określenie wpływu aldehydu *trans*-cynamonowego na wzrost, przeżywalność oraz cechy związane z wirulencją szczepów *E. coli*.
4. Ocena wpływu *t*-CA na ekspresję toksyny Shiga oraz rozwój bakteriofagów niosących geny *stx*.
5. Określenie znaczenia odpowiedzi ścisłej w mechanizmie działania aldehydu *trans*-cynamonowego.
6. Identyfikacja zmian metabolicznych i transkryptomicznych zachodzących w komórkach *E. coli* pod wpływem *t*-CA.
7. Ocena wpływu *t*-CA na tworzenie biofilmu oraz adaptację bakterii do warunków stresowych.
8. Ocena potencjalnego synergistycznego działania *t*-CA w połączeniu z wybranymi antybiotykami.
9. Ocena aktywności biologicznej i potencjału antywirulencyjnego *t*-CA w modelach *in vivo*.

Realizacja przedstawionych celów szczegółowych pozwoliła na wszechstronną ocenę działania aldehydu *trans*-cynamonowego wobec patogennych szczepów *E. coli*, obejmującą zarówno efekty przeciwdrobnoustrojowe i antywirulencyjne, jak i molekularne podstawy obserwowanych zjawisk.

8. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

PUBLIKACJA 1.

*„Antibacterial activity of *t*-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)”*

Narastająca częstość zakażeń trudnych do leczenia oraz problem antybiotykooporności podkreślają potrzebę poszukiwania nowych strategii przeciwdrobnoustrojowych. Substancje pochodzenia naturalnego są obiecującą grupą związków o potencjalnym zastosowaniu w zwalczaniu zakażeń bakteryjnych jednak mechanizmy odpowiedzialne za ich aktywność biologiczną często pozostają nie jasne. Jednym z takich związków jest aldehyd *trans*-cynamonowy. W celu poznania jego działania do badań wykorzystano enterokrwotoczne szczepy *E. coli* (EHEC), które stanowią szczególnie użyteczny model do analizy regulacji wirulencji bakterii. Szczepy te produkują toksynę Shiga odpowiedzialną za rozwój ciężkich powikłań zakażenia, w tym zespołu hemolityczno-mocznicowego. Ekspresja genów *stx*, kodujących toksynę, jest bezpośrednio związana z rozwojem bakteriofagów lambdoidalnych występujących w komórkach w postaci profagów. Proces ten może być indukowany przez czynniki stresowe, w tym aktywujące odpowiedź SOS, jak niektóre antybiotyki, co ogranicza możliwości strategii terapeutyczne zakażeń związanych z EHEC.

Początkowo oceniono wpływ *t*-CA na wzrost szczepów EHEC oraz szczepu laboratoryjnego MG1655. Wykazano, że badany związek działa bakteriobójczo wobec wszystkich analizowanych szczepów, jednak szczepy EHEC wykazywały większą wrażliwość na jego działanie niż szczep K-12. Wartości minimalnego stężenia hamującego (MIC) mieściły się w zakresie 1,875–3,750 mM, natomiast minimalnego stężenia bakteriobójczego (MBC) w zakresie 3,75–7,50 mM. Analiza kinetyki zabijania (*time kill*) wykazała szybkie ograniczenie liczby żywych komórek bakteryjnych już po kilku godzinach inkubacji z *t*-CA, natomiast pomiary aktywności metabolicznej z wykorzystaniem systemu bioluminescencyjnego wskazywały na gwałtowne obniżenie aktywności komórkowej pod wpływem badanego związku.

Obserwowane zahamowanie wzrostu było powiązane z wyraźnymi zmianami strukturalnymi komórek bakteryjnych. Doświadczenia wykazały deformację komórek, ich obkurczenie, oddzielenie błony komórkowej od ściany oraz kondensację cytoplazmy. Zmianom tym towarzyszyło zwiększenie przepuszczalności błony komórkowej, co

wskazywało na utratę jej integralności i zaburzenie homeostazy komórki prowadzące ostatecznie do jej śmierci.

W celu dokładniejszego scharakteryzowania odpowiedzi komórki na działanie *t*-CA przeprowadzono analizę transkryptomiczną RNA-seq. Ekspozycja bakterii na subinhibitorowe stężenie aldehydu prowadziła do szybkich i rozległych zmian ekspresji genów. Po 30 minutach obserwowano nadekspresję około 292 genów oraz wyciszenie 107 genów, natomiast po 60 minutach odpowiednio 140 i 96 genów wykazywało zmienioną ekspresję. Analiza funkcjonalna GO i KEGG wykazała, że zmiany te obejmowały przede wszystkim centralny metabolizm komórki i procesy energetyczne. Obniżona była ekspresja genów związanych z glikolizą, cyklem TCA oraz metabolizmem kwasów tłuszczowych, co wskazywało na zahamowanie podstawowych szlaków produkcji energii i przejście komórki w stan stresu metabolicznego.

Jednocześnie obserwowano aktywację alternatywnych szlaków energetycznych, w tym nadekspresję genów operonów *tor* oraz *nar* związanych z oddychaniem beztlenowym i redukcją azotanów. Stwierdzono także liczne zmiany w ekspresji genów regulatorowych związanych z odpowiedzią na stres oraz utrzymaniem równowagi redoks. Szczególnie istotna okazała się silna nadekspresja genu *poxB* kodującego oksydazę pirogronianową prowadzącą do produkcji octanu i zakwaszenia cytoplazmy. Również jednym z najsilniej indukowanych genów był *cadA* kodujący dekarboksylazę lizynową uczestniczącą w odpowiedzi na stres kwasowy. Zwiększona aktywność CadA pozbawia komórkę lizyny, co zaobserwowano na podstawie wzrostu stężenia kadaweryny w komórkach po podaniu *t*-CA.

W związku z obserwowanymi zmianami metabolicznymi postanowiono zbadać wpływ *t*-CA na procesy biosyntetyczne komórki. W komórkach typu dzikiego dochodziło do szybkiego zahamowania syntezy zarówno DNA, jak i RNA, podczas gdy efekt ten nie występował w szczepach z delecjami genów *relA* oraz *relAspoT*. Ponieważ podobne zahamowanie procesów biosyntetycznych jest charakterystycznym elementem odpowiedzi ścisłej a wcześniejsze badania wykazały, że odpowiedź ścisła zależna od alarmonów (p)ppGpp i może ograniczać rozwój fagów Stx oraz hamować ekspresję toksyny Shiga, postanowiono w kolejnym etapie przeanalizować wpływ *t*-CA na poziom alarmonów (p)ppGpp. Wykazano, że ekspozycja bakterii na aldehyd prowadziła do zależnej od RelA akumulacji alarmonów, co wskazywało na aktywację odpowiedzi ścisłej oraz globalne przeprogramowanie funkcjonowania komórki bakteryjnej.

Istotnym elementem pracy była również analiza wpływu *t*-CA na stres oksydacyjny, ponieważ wiele antybiotyków indukuje rozwój bakteriofagów Stx poprzez zwiększenie poziomu reaktywnych form tlenu i aktywację odpowiedzi SOS. W komórkach poddanych działaniu *t*-CA nie obserwowano jednak wzrostu poziomu ROS ani zwiększenia poziomu donorów reaktywnych form tlenu, takich jak NAD/NADH. Analiza RNA-seq nie wykazała również istotnych zmian w ekspresji genów związanych z odpowiedzią SOS, takich jak *recA*, *lexA* czy *sulA*. Uznano, że działanie aldehydu *trans*-cynamonowego nie prowadzi do indukcji klasycznej odpowiedzi na uszkodzenia DNA ani stresu oksydacyjnego charakterystycznego dla wielu antybiotyków.

Mając na uwadze obserwowaną aktywację odpowiedzi ścisłej oraz brak indukcji odpowiedzi SOS, oceniono wpływ *t*-CA na rozwój bakteriofagów niosących geny *stx* oraz ekspresję toksyny Shiga. Wykazano, że w obecności aldehydu dochodziło do zahamowania rozwoju fagów nawet w warunkach silnej indukcji. Efekt ten był zależny od funkcjonowania odpowiedzi ścisłej, ponieważ w szczepach z delecją *relA* obserwowano odmienny przebieg tego procesu. W konsekwencji dochodziło również do obniżenia ekspresji genów toksyny Shiga oraz ograniczenia potencjału wirulencyjnego szczepów EHEC.

Znaczenie biologiczne uzyskanych wyników potwierdzono w modelu *G. mellonella*. Podanie larwom *t*-CA po zakażeniu szczepami EHEC prowadziło do istotnego, zależnego od dawki zwiększenia przeżywalności. Najwyższe dawki związku zapewniały najsilniejszy efekt ochronny bez wykazywania toksycznego wpływu na larwy niezainfekowane. Dodatkowo wykazano ograniczenie ekspresji toksyny Shiga w organizmie gospodarza oraz korzystne współdziałanie *t*-CA z azytromycyną, prowadzące do silniejszego zahamowania ekspresji toksyny niż w przypadku działania każdego z tych związków oddzielnie.

Uzyskane wyniki wskazywały, że aldehyd *trans*-cynamonowy działa wielokierunkowo i jego aktywność wykracza poza klasyczny efekt przeciwdrobnoustrojowy. Oprócz hamowania wzrostu bakterii i zaburzenia integralności komórki prowadził również do szerokich zmian metabolicznych, aktywacji odpowiedzi ścisłej, ograniczenia rozwoju fagów Stx oraz obniżenia ekspresji toksyny Shiga. Obserwacje te sugerowały, że działanie antywirulencyjne *t*-CA jest związane z globalnym przeprogramowaniem funkcjonowania komórki bakteryjnej i stały się podstawą do dalszych badań nad molekularnym mechanizmem działania tego związku.

PUBLIKACJA 2.

„Increased Levels of (p)ppGpp correlate with virulence and biofilm formation, but not with growth, in strains of uropathogenic Escherichia coli”

Uzyskane wcześniej wyniki badań dotyczące aktywności *t*-CA wobec szczepów EHEC, a w szczególności jego wpływ na kontrolę wirulencji, skłoniły do rozszerzenia badań o kolejny model patogennych szczepów *E. coli*. W tym celu do badań włączono uropatogenne szczepy *E. coli*.

Celem prac przedstawionych w tej publikacji była charakterystyka kolekcji klinicznych szczepów UPEC izolowanych od pacjentów z zakażeniami układu moczowego hospitalizowanych w Wojewódzkim Szpitalu w Gdańsku. Szczepy UPEC należą do najważniejszych czynników etiologicznych ZUM, a skuteczność leczenia tych zakażeń jest dodatkowo ograniczana przez narastającą antybiotykooporność oraz zdolność bakterii do tworzenia biofilmu. Pomimo licznych badań nadal nie w pełni poznane pozostają zależności pomiędzy profilem wirulencji, lekoopornością oraz właściwościami fenotypowymi szczepów klinicznych mogącymi sprzyjać utrzymywaniu się zakażenia i jego nawrotom.

Badaniami objęto kolekcję 120 szczepów UPEC, w tym 118 izolatów klinicznych poddanych charakterystyce oraz dwa szczepy referencyjne wykorzystane do celów porównawczych. Celem pracy było określenie wybranych cech genotypowych i fenotypowych szczepów *E. coli* istotnych dla przebiegu procesu zakażenia. Analiza wykazała znaczną różnorodność badanej populacji zarówno pod względem przynależności filogenetycznej, jak i obecności genów wirulencji. Identyfikowano m.in. geny kodujące adhezyny (*fimH*, *sfa*, *papC*), czynniki toksyczne (*hlyA*, *cnf1*), elementy związane z inwazją (*ibeA*), systemy pozyskiwania żelaza (*aer*, *sitA*), a także geny związane z przeżyciem w surowicy i tworzeniem biofilmu (*iss*, *agn43*). Analiza czynników wirulencji wykazała, że wszystkie badane szczepy posiadały gen *fimH*, natomiast geny *sitA*, *aer* oraz *sfa* należały do najczęściej występujących dodatkowych czynników wirulencji. Poszczególne izolaty zawierały różne kombinacje tych genów i łącznie zidentyfikowano 51 odmiennych wirulotypów co podkreśla wysoką heterogenność badanej kolekcji. Większość izolatów (95,8%) zawierała jednocześnie kilka genów związanych z patogennością, co wskazuje na obecność rozbudowanego repertuaru determinant umożliwiających skuteczną kolonizację dróg moczowych

i utrzymywanie się bakterii w organizmie gospodarza. W badanej kolekcji dominowała filogrupa B2 (71,7%), najczęściej kojarzona z zakażeniami pozajelitowymi oraz zwiększonym potencjałem wirulencji. Mniejszy udział stanowiły grupy D, A oraz B1.

Istotnym elementem pracy była analiza zdolności tworzenia biofilmu. Około jedna trzecia ($n = 39$) badanych szczepów wykazywała fenotyp biofilmotwórczy na podłożu Congo Red Agar. Jednocześnie nie stwierdzono zależności pomiędzy zdolnością tworzenia biofilmu a przynależnością do określonej grupy filogenetycznej, co sugeruje, że cecha ta nie jest bezpośrednio związana z pochodzeniem filogenetycznym szczepów. Natomiast szczepy tworzące biofilm częściej wykazywały oporność na antybiotyki, w tym oporność wielolekową. Zmniejszoną wrażliwość na co najmniej jeden antybiotyk stwierdzono u 44,2% analizowanych izolatów, a znaczna część szczepów wykazywała oporność na więcej niż lek. Wyniki te wskazują, że zdolność tworzenia biofilmu może współwystępować z cechami zwiększającymi przeżywalność bakterii w warunkach presji środowiskowej i antybiotykowej.

W związku z udokumentowaną rolą alarmonów (p)ppGpp w regulacji tempa wzrostu, procesów adaptacyjnych i ekspresji cech wirulencji przeanalizowano, czy szczepy UPEC różniące się zdolnością do tworzenia biofilmu wykazują również odmienne poziomy tych alarmonów. Wykazano, że szczepy zdolne do tworzenia biofilmu utrzymywały podwyższony podstawowy poziom (p)ppGpp w porównaniu ze szczepami niewytwarzającymi biofilmu. Co istotne, szczepy te wykazywały również większą wirulencję w infekcyjnym modelu wykorzystującym larwy barciaka większego, *G. mellonella*. Uzyskane obserwacje sugerują możliwy związek odpowiedzi ściślej z regulacją procesów związanych z adaptacją bakterii do warunków środowiskowych występujących podczas zakażenia.

Przeprowadzone analizy pozwoliły wykazać współwystępowanie cech związanych z wirulencją, biofilmem oraz wielolekoopornością w obrębie klinicznych szczepów UPEC i wskazały, że szczepy biofilmotwórcze mogą reprezentować szczególny fenotyp sprzyjający przewlekłości oraz nawrotowości zakażeń układu moczowego.

PUBLIKACJA 3.

„Trans-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic Escherichia coli.”

W kolejnym etapie badań rozszerzono analizę działania aldehydu *trans*-cynamonowego na inne patogenne szczepy *E. coli*, w szczególności szczepy pozajelitowe (ExPEC). Uzyskane wcześniej wyniki sugerowały, że aktywność *t*-CA nie ogranicza się wyłącznie do bezpośredniego hamowania wzrostu bakterii, lecz może obejmować również procesy związane z regulacją wirulencji i odpowiedzią komórki na stres. W związku z tym podjęto badania mające na celu lepsze poznanie mechanizmów odpowiedzialnych za biologiczne działanie tego związku, ze szczególnym uwzględnieniem udziału odpowiedzi ścisłej, zmian w funkcjonowaniu komórki bakteryjnej oraz wpływu *t*-CA na cechy związane z patogennością bakterii.

Początkowo przeprowadzono ocenę wrażliwości 145 klinicznych i środowiskowych izolatów *E. coli* na działanie *t*-CA. Badana kolekcja obejmowała zarówno szczepy jelitowe, jak i pozajelitowe, w tym liczną grupę klinicznych szczepów uropatogennych (n=120) charakteryzujących się znacznym zróżnicowaniem pod względem cech wirulencji, zdolności tworzenia biofilmu oraz oporności na antybiotyki. Uzyskane wyniki wykazały stosunkowo wyrównaną podatność analizowanych szczepów na działanie *t*-CA, a wartość MIC₉₀ wynosiła 3,75 mM. W celu określenia potencjalnego udziału odpowiedzi ścisłej w mechanizmie działania badanego związku analizą objęto również szczepy pozbawione funkcjonalnych genów *relA* oraz *spoT*. Wykazywały one zwiększoną wrażliwość na działanie aldehydu *trans*-cynamonowego w porównaniu ze szczepem typu dzikiego, co sugerowało istotną rolę odpowiedzi ścisłej w adaptacji bakterii do stresu wywoływanego przez *t*-CA.

Wykorzystując tak scharakteryzowaną kolekcję szczepów oceniono wpływ *t*-CA na biofilm. Wykazano, że ekspozycja na związek prowadziła do wyraźnego zaburzenia jego struktury – zwarte agregaty komórek ulegały rozpadowi, a dominującą formą stawały się komórki rozproszone. Analiza ilościowa przeprowadzona dla całości kolekcji 120 szczepów UPEC wykazała istotne, zależne od dawki zmniejszenie masy biofilmu, czemu towarzyszyło obniżenie aktywności metabolicznej komórek. Jednocześnie obserwowano zahamowanie produkcji kluczowych składników macierzy biofilmu, w tym włókien amyloidowych (curli) oraz celulozy, co wskazuje na zaburzenie procesów odpowiedzialnych za jego tworzenie i stabilność.

Porównanie profilu transkrypcyjnego szczepu WT i mutantu $\Delta relA$ wykazało, że obecność RelA wpływała nie tylko na zakres, ale również na kierunek odpowiedzi na *t*-CA. W komórkach typu dzikiego zmiany miały charakter bardziej uporządkowany i czasowo skoordynowany, natomiast w mutantach $\Delta relA$ były znacznie bardziej rozproszone, co sugeruje, że RelA pełni funkcję koordynującą odpowiedź transkrypcyjną. Pod wpływem działania *t*-CA dochodziło do zahamowania kluczowych szlaków wirulencji takich jak quorum sensing oraz elementy związane z ruchliwością i kolonizacją. Jednocześnie obserwowano ograniczenie aktywności procesów energetycznie kosztownych, co wskazuje na przeprogramowanie funkcjonowania komórki w kierunku oszczędniejszego gospodarowania zasobami metabolicznymi. Profil tych zmian jest zgodny z cechami charakterystycznymi dla odpowiedzi ścisłej, w której dochodzi do ograniczenia procesów związanych ze wzrostem przy jednoczesnym zwiększeniu zdolności adaptacyjnych komórki. Mimo że *t*-CA nadal hamował wzrost bakterii $\Delta relA$, komórki nie były w stanie prawidłowo przeprogramować ekspresji genów. Zamiast wyciszenia procesów wzrostowych, dochodziło do nadekspresji genów związanych z translacją (białka rybosomalne), biosyntezą oraz ruchem (flagelle). Oznacza to, że komórki pozostawały w stanie gotowości do wzrostu i nie były w stanie właściwie odpowiedzieć na sygnał stresowy, co prowadziło do nieskoordynowanej i nieefektywnej odpowiedzi transkrypcyjnej.

Uzyskane wyniki dotyczące aktywności przeciwdrobnoustrojowej *t*-CA oraz jego wpływu na procesy regulacyjne komórki bakteryjnej skłoniły do oceny działania związku w modelach eukariotycznych, umożliwiających jednoczesną ocenę jego bezpieczeństwa i skuteczności. Wpływ *t*-CA na komórki eukariotyczne oraz przebieg zakażenia oceniono w modelach *in vitro* i *in vivo*. W liniach komórek nabłonkowych HB2 oraz fibroblastów HDFa wykazano zależny od stężenia spadek żywotności po 24-godzinnej ekspozycji na *t*-CA, przy czym komórki HB2 były bardziej wrażliwe ($IC_{50} = 47,6 \mu M$) niż fibroblasty HDFa ($IC_{50} = 102,8 \mu M$). Pomimo tych różnic nie obserwowano gwałtownej cytotoksyczności w badanym zakresie stężeń, co wskazuje na stopniowy wpływ związku na aktywność metaboliczną komórek. Podobny zakres wrażliwości wykazano dla komórek PBMC (*Peripheral Blood Mononuclear Cells*, komórki jednojądrzaste krwi obwodowej) ($IC_{50} = 67,2 \mu M$), w których jednocześnie obserwowano zależną od dawki modulację odpowiedzi immunologicznej, obejmującą obniżenie poziomu cytokin prozapalnych (IL-1 β , TNF- α , IL-6) oraz tendencję do zwiększonego wydzielania IL-10. Na tle tych obserwacji oceniono działanie *t*-CA w modelu *in vivo* *G. mellonella*.

W modelu zakażenia szczepem UPEC-UTI89 zakażone larwy kontrolne wykazywały szybki spadek przeżywalności, podczas gdy podanie *t*-CA po infekcji prowadziło do istotnego, zależnego od dawki zwiększenia ich przeżywalności. Najwyższe dawki (25–50 mg/kg masy ciała) zapewniały najsilniejszy efekt ochronny, przy jednoczesnym braku toksycznego wpływu na larwy niezainfekowane. Uzyskane wyniki wskazują, że aktywność biologiczna *t*-CA obserwowana w warunkach *in vitro* przekłada się również na efekt ochronny w modelu zakażenia *in vivo*, co podkreśla potencjalne znaczenie terapeutyczne tego związku.

PUBLIKACJA 4 (nierecenzowany manuskrypt)

„Spicing up urinary tract infections: synergistic action of fosfomycin with trans-cinnamaldehyde - insight to the mode of action”

W poprzednich etapach badań wykazano, że aldehyd *trans*-cynamonowy wpływa na funkcjonowanie komórki bakteryjnej na wielu poziomach, obejmujących zarówno procesy metaboliczne, jak i cechy związane z wirulencją. Uzyskane wyniki wskazywały, że *t*-CA nie działa wyłącznie jako związek przeciwdrobnoustrojowy, lecz prowadzi do głębokiej przebudowy funkcjonowania bakterii, wpływając na ich zdolność do adaptacji do stresu oraz utrzymywania fenotypu patogennego. Naturalnym pytaniem stało się więc czy taki związek może zwiększać skuteczność istniejących antybiotyków i znaleźć praktyczne zastosowanie terapeutyczne.

Przeprowadzona analiza interakcji aldehydu *trans*-cynamonowego z przedstawicielami różnych klas leków przeciwbakteryjnych wykazała, że najsilniejszy oraz najbardziej powtarzalny efekt synergistyczny obserwowano w skojarzeniu z fosfomycyną. Wynik ten zasługuje na szczególną uwagę, ponieważ fosfomycyna należy do kluczowych antybiotyków stosowanych w terapii zakażeń układu moczowego, a jej skuteczność jest w znacznym stopniu uzależniona od stanu fizjologicznego i aktywności metabolicznej komórek bakteryjnych. Dalsze badania potwierdziły, że obecność *t*-CA istotnie zwiększa aktywność przeciwbakteryjną fosfomycyny, umożliwiając skuteczne hamowanie wzrostu bakterii przy niższych stężeniach obu związków. Ponadto wykazano, że skojarzone działanie tych substancji prowadzi do nasilenia efektu bakteriobójczego w porównaniu z zastosowaniem każdego z nich osobno. Szczególnie istotne okazały się wyniki dotyczące oddziaływania badanych związków na biofilm bakteryjny, uznawany za jeden z kluczowych czynników warunkujących przewlekły i nawrotowy charakter zakażeń wywoływanych przez uropatogenne szczepy *E. coli*. Wykazano, że zastosowanie terapii skojarzonej prowadzi do istotnego ograniczenia żywotności oraz aktywności metabolicznej komórek zasiedlających biofilm. Jednocześnie zaobserwowano zaburzenia jego architektury przestrzennej, redukcję ilości macierzy zewnątrzkomórkowej oraz ograniczenie zdolności bakterii do kolonizacji powierzchni cewników. Uzyskane wyniki wskazują, że aldehyd *trans*-cynamonowy nie tylko nasila aktywność przeciwbakteryjną fosfomycyny, lecz także osłabia struktury i mechanizmy odpowiedzialne za utrzymywanie się drobnoustrojów w organizmie gospodarza, co może przyczyniać się do zwiększenia skuteczności terapii oraz ograniczenia ryzyka nawrotów zakażenia.

W celu wyjaśnienia mechanizmów leżących u podstaw obserwowanego efektu synergistycznego przeprowadzono badania ukierunkowane na analizę procesów metabolicznych. Podejście to było szczególnie uzasadnione w przypadku fosfomycyny, będącej analogiem fosfoenolopirogronianu (PEP), której aktywność przeciwbakteryjna pozostaje ściśle związana ze stanem metabolicznym komórki. Jednocześnie wcześniejsze badania wykazały, że ekspozycja bakterii na aldehyd *trans*-cynamonowy prowadzi do zmian w obrębie centralnego metabolizmu węgla, obejmujących między innymi zwiększoną ekspresję genu *poxB* kodującego oksydazę pirogronianową, co wskazywało na istnienie zależności pomiędzy działaniem *t*-CA a przemianami metabolicznymi zachodzącymi w obrębie węzła obejmującego fosfoenolopirogronian i pirogronian. Badania dokowania molekularnego wykazały, że spośród analizowanych białek związanych z przemianami pirogronianu najbardziej biologicznie uzasadnione było potencjalne oddziaływanie *t*-CA z podjednostką AceE kompleksu dehydrogenazy pirogronianowej, ponieważ przewidywane miejsce wiązania związku lokalizowało się w pobliżu regionu katalitycznego. Jednocześnie pomiar wewnątrzkomórkowego poziomu pirogronianu wykazał jego obniżenie po ekspozycji bakterii na *t*-CA, co wskazywało na zaburzenie homeostazy tego metabolitu oraz możliwą modyfikację przepływu węgla przez centralne szlaki metaboliczne. Dodatkowe analizy z wykorzystaniem szczepów delecyjnych w podjednostkach kompleksu dehydrogenazy pirogronianowej (PDH) (*aceE*, *aceF*, *lpdA*) potwierdziły, iż dysregulacja metabolizmu pirogronianu w komórce znacząco uwrażliwia szczepy na działanie *t*-CA. To odkrycie mocno uprawdopodobnia PDH jako cel molekularny *t*-CA. Ponadto, dodatkowych przesłanek dostarczyły eksperymenty z wykorzystaniem układu BtsSR odpowiedzialnego za wykrywanie pirogronianu. Wykazano, że zarówno obecność *t*-CA, jak i egzogenne pirogronianu wpływała na fenotyp kolonii na podłożu Congo Red, podczas gdy efekt ten nie występował w szczepie pozbawionym funkcjonalnego układu BtsSR. Obserwacje te wskazują na udział procesów związanych z metabolizmem i sygnalizacją pirogronianową w odpowiedzi bakterii na działanie aldehydu *trans*-cynamonowego. Chociaż uzyskane wyniki nie pozwalają na jednoznaczne określenie molekularnych podstaw synergistycznego działania *t*-CA i fosfomycyny, dostarczają silnych przesłanek wskazujących, że aldehyd *trans*-cynamonowy wpływa na centralny metabolizm bakterii i może modulować procesy istotne dla skuteczności działania fosfomycyny.

Ze względu na narastający problem oporności bakterii na antybiotyki szczególnie ważna była ocena wpływu aldehydu na proces adaptacji bakterii do działania fosfomycyny. Uzyskane wyniki wykazały, że obecność *t*-CA ogranicza tempo nabywania oporności na ten antybiotyk

podczas długotrwałej ekspozycji. W przeciwieństwie do hodowli prowadzonych wyłącznie w obecności fosfomycyny, dodatek *t*-CA istotnie spowalniał wzrost wartości minimalnego stężenia hamującego, nie prowadząc jednocześnie do selekcji szczepów o obniżonej wrażliwości na sam aldehyd. Wyniki te sugerują, że korzyści wynikające ze stosowania terapii skojarzonej mogą obejmować nie tylko zwiększenie aktywności przeciwbakteryjnej, lecz również ograniczenie procesów sprzyjających rozwojowi i utrwalaniu oporności.

Ostatecznego potwierdzenia biologicznego znaczenia obserwowanych efektów synergistycznych dostarczyły badania przeprowadzone z wykorzystaniem modelu zakażenia *G. mellonella*. Wykazano, że terapia skojarzona zapewniała skuteczniejszą ochronę gospodarza niż każdy z badanych związków stosowany oddzielnie. Przeprowadzone badania stanowią rozwinięcie dotychczasowej wiedzy dotyczącej biologicznej aktywności aldehydu *trans*-cynamonowego oraz rozszerzają ją o aspekty związane z jego oddziaływaniem z antybiotykami. Wykazano, że związek ten może nie tylko modulować metabolizm bakterii i osłabiać mechanizmy związane z ich wirulencją, lecz także zwiększać aktywność wybranych leków przeciwbakteryjnych oraz ograniczać tempo nabywania oporności.

W świetle uzyskanych wyników aldehyd *trans*-cynamonowy można uznać za obiecujący związek wspomagający działanie konwencjonalnych antybiotyków. Przedstawione badania wskazują, że fitozwiązki tego typu mogą stanowić wartościowy element strategii ukierunkowanych na zwiększenie skuteczności terapii zakażeń wywoływanych przez patogenne szczepy *E. coli* oraz ograniczać narastanie oporności na leki przeciwbakteryjne.

PUBLIKACJA 5.

„Phytochemicals controlling enterohemorrhagic Escherichia coli (EHEC) virulence-current knowledge of their mechanisms of action”

Praca przeglądowa została poświęcona naturalnym związkom wykazującym aktywność wobec enterokrwotocznym szczepom *E. coli*. Jej celem było zebranie i usystematyzowanie aktualnej wiedzy dotyczącej fitozwiązków wpływających na wirulencję tego patogenu, ze szczególnym uwzględnieniem molekularnych mechanizmów ich działania. Potrzeba poszukiwania nowych metod ograniczania zakażeń EHEC wynika z faktu, że stosowanie niektórych antybiotyków może prowadzić do zwiększonej produkcji toksyn Shiga, odpowiedzialnych za rozwój ciężkich powikłań.

W pracy przedstawiono charakterystykę najważniejszych czynników wirulencji EHEC, obejmujących produkcję toksyn Shiga, funkcjonowanie regionu LEE odpowiedzialnego za kolonizację nabłonka jelitowego oraz tworzenie biofilmu. Następnie omówiono aktualne doniesienia dotyczące różnych grup fitozwiązków, w tym izotiocyjanianów, związków fenolowych oraz terpenów, dla których opisano aktywność przeciwwirulentną wobec EHEC. Szczególną uwagę poświęcono mechanizmom związanym z hamowaniem produkcji toksyn Shiga, ograniczaniem indukcji bakteriofagów niosących geny *stx*, hamowaniem tworzenia biofilmu oraz zmniejszaniem zdolności bakterii do kolonizacji gospodarza.

Przegląd wykazał, że liczne związki pochodzenia roślinnego mogą skutecznie oddziaływać na kluczowe procesy warunkujące patogenność EHEC, nie działając wyłącznie bakteriobójczo. Podsumowanie dostępnych danych wskazuje, że fitozwiązki stanowią obiecującą grupę substancji mogących znaleźć zastosowanie w nowych strategiach przeciwwirulentnych ukierunkowanych na ograniczanie zakażeń wywołanych przez EHEC. Jednak wiele aspektów ich działania pozostaje nadal niewystarczająco poznanych. Szczególnie ważne wydaje się dalsze wyjaśnienie molekularnych mechanizmów odpowiedzialnych za obserwowane efekty przeciwwirulencyjne oraz identyfikacja komórkowych celów działania poszczególnych związków. Praca przeglądowa ukazuje badania nad aldehydem *trans*-cynamonowym, będącym przedmiotem tej rozprawy, na tle innych związków pochodzenia roślinnego stosowanych do zwalczania infekcji EHEC. Lepsze zrozumienie zależności pomiędzy strukturą chemiczną fitozwiązków a ich aktywnością biologiczną może przyczynić się do opracowania skuteczniejszych strategii ograniczania zakażeń. Konieczne są również dalsze badania oceniające skuteczność tych związków w bardziej złożonych modelach biologicznych oraz warunkach zbliżonych do naturalnego przebiegu zakażenia

9. PODSUMOWANIE

Przeprowadzone badania pozwoliły na kompleksowe scharakteryzowanie biologicznego działania aldehydu *trans*-cynamonowego wobec patogennych szczepów *Escherichia coli* oraz określenie mechanizmów odpowiedzialnych za obserwowane efekty przeciwdrobnoustrojowe i antywirulencyjne. Kolejne prace koncentrowały się na różnych aktywnościach biologicznych badanego związku, prowadząc do wyjaśnienia mechanizmów leżących u podstaw jego działania.

W pracach wykazałam, że aldehyd *trans*-cynamonowy oddziałuje na komórkę bakteryjną wywołując zmiany obejmujące zarówno procesy metaboliczne, jak i cechy związane z wirulencją oraz adaptacją bakterii do warunków stresowych. Wykazałam, że działanie *t*-CA nie ogranicza się wyłącznie do efektu hamowania wzrostu bakterii, lecz prowadzi do globalnej przebudowy funkcjonowania komórki bakteryjnej, obejmującej zaburzenie homeostazy metabolicznej, zahamowanie procesów biosyntetycznych czy ograniczenie tworzenia biofilmu.

Istotnym było również wykazanie, że aldehyd *trans*-cynamonowy osłabia mechanizmy związane z patogennością bakterii EHEC. Obserwowane efekty dotyczyły między innymi ograniczenia ekspresji toksyny Shiga, zahamowania rozwoju bakteriofagów niosących geny *stx*, zmniejszenia zdolności tworzenia biofilmu oraz przebudowy szlaków metabolicznych związanych z adaptacją bakterii do stresu. Szczególne znaczenie ma fakt, że *t*-CA nie indukował odpowiedzi SOS ani wzrostu poziomu reaktywnych form tlenu, co odróżnia jego działanie od wielu klasycznych czynników stresowych i części antybiotyków mogących nasilać wirulencję szczepów EHEC. Ważnym elementem badań było wskazanie udziału odpowiedzi ścisłej w mechanizmie działania *t*-CA. Uzyskane dane pokazały, że ekspozycja bakterii na *t*-CA prowadzi do aktywacji procesów związanych z odpowiedzią zależną od RelA, czemu towarzyszą zmiany obejmujące metabolizm komórki, procesy biosyntetyczne oraz globalną reorganizację profilu transkrypcyjnego. Analizy porównawcze szczepu typu dzikiego i mutantów pozbawionych funkcjonalnych elementów odpowiedzi ścisłej wykazały, że mechanizmy zależne od RelA uczestniczą w koordynacji odpowiedzi komórki bakteryjnej na działanie badanego związku. Pozwoliło to powiązać obserwowane efekty antywirulencyjne i metaboliczne z funkcjonowaniem jednego z najważniejszych bakteryjnych układów adaptacyjnych.

Istotnym rozszerzeniem przedstawionych badań było wykazanie, że konsekwencje metaboliczne działania aldehydu *trans*-cynamonowego mogą przekładać się na zwiększenie

skuteczności terapii przeciwbakteryjnej. Wykazano, że *t*-CA nasila aktywność fosfomicyny wobec uropatogennych szczepów *E. coli*, zwiększając jej skuteczność wobec komórek planktonowych i biofilmowych oraz ograniczając tempo nabywania oporności podczas długotrwałej ekspozycji bakterii na antybiotyki. Uzyskane wyniki wskazują, że oddziaływanie *t*-CA na centralny metabolizm komórki bakteryjnej może mieć znaczenie nie tylko dla regulacji wirulencji, lecz również dla modulowania wrażliwości bakterii na działanie leków przeciwbakteryjnych.

W pracy wykorzystano różnorodne modele badawcze oraz metody eksperymentalne. Badania obejmowały analizę klinicznych i środowiskowych szczepów *E. coli*, modele genetyczne: mutanty regulatorów odpowiedzi ścisłej i wybranych szlaków metabolicznych, a także modele komórkowe oraz modele zakażenia *in vivo*. Do oceny aktywności badanego związku wykorzystano metody pozwalające na analizę zmian fenotypowych, metabolicznych i transkrypcyjnych bakterii. Szerokie podejście metodologiczne pozwoliło nie tylko na potwierdzenie obserwowanych efektów w różnych układach eksperymentalnych, lecz również na zaproponowanie hipotezy dotyczącej mechanizmu działania *t*-CA w komórce bakteryjnej.

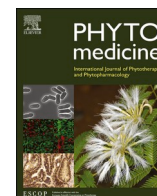
Łącznie uzyskane wyniki wskazują, że aldehyd *trans*-cynamonowy działa jako wielopoziomowy modulator funkcjonowania komórki bakteryjnej, obejmując swoim działaniem zarówno procesy związane z przeżywalnością bakterii, jak i mechanizmy odpowiedzialne za utrzymywanie ich potencjału patogenego. Przedstawiony cykl prac dostarczył obrazu zależności pomiędzy stanem metabolicznym, odpowiedzią ścisłą i procesami związanymi z wirulencją, a także poszerzył aktualną wiedzę dotyczącą biologicznego działania *t*-CA oraz wskazał na potencjalne zastosowanie zarówno w strategiach antywirulencyjnych, jak i w terapiach wspomagających działanie antybiotyków.

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11. PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ



Original Article

Antibacterial activity of *t*-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)

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ARTICLE INFO

Keywords:

Antimicrobials
Bacteriophage
Cinnamaldehyde
G. mellonella
O157:H7
ppGpp

ABSTRACT

Background: Compounds of natural origin are potent source of drugs with unique mechanisms of action. Among phytochemicals, *trans*-cinnamaldehyde (*t*-CA) exhibits a wide range of biological activity, thus has been used for centuries to fight bacterial and fungal infections. However, the molecular basis of these properties has not been fully covered. Considering that difficult-to-control infections are becoming a rising global problem, there is a need to elucidate the molecular potential of *t*-CA.

Purpose: To evaluate the antibacterial activity of *t*-CA against Shiga-toxigenic *E. coli* strains and elucidate its mechanism of action based on the inhibition of the virulence factor expression.

Methods: The antimicrobial potential of *t*-CA was assessed with two-fold microdilution and time-kill assays. Further evaluation included bioluminescence suppression assays, quantification of reactive oxygen species (ROS) and assessment of NAD⁺/NADH ratios. Morphological changes post *t*-CA exposure were examined using transmission electron microscopy. RNA sequencing and radiolabeling of nucleotides elucidated the metabolic alterations induced by *t*-CA. Toxin expression level was monitored through the application of fusion proteins, monitoring of bacteriophage development, and fluorescence microscopy studies. Lastly, the therapeutic efficacy *in vivo* was assessed using *Galleria mellonella* infection model.

Results: A comprehensive study of *t*-CA's bioactivity showed unique properties affecting bacterial metabolism and morphology, resulting in significant bacterial cell deformation and effective virulence inhibition. Elucidation of the underlying mechanisms indicated that *t*-CA activates the global regulatory system, the stringent response, manifested by its alarmone, (p)ppGpp, overproduction mediated by the RelA enzyme, thereby inhibiting bacterial proliferation. Intriguingly, *t*-CA effectively downregulates Shiga toxin gene expression via alarmone molecules, indicating its potential for therapeutic effect. *In vivo* validation demonstrated a significant improvement in larval survival rates post- *t*-CA treatment with 50 mg/kg ($p < 0.05$), akin to the efficacy observed with azithromycin, thus indicating its effectiveness against EHEC infections ($p < 0.05$).

Conclusions: Collectively, these results reveal the robust antibacterial capabilities of *t*-CA, warranting its further exploration as a viable anti-infective agent.

Introduction

The global emergence of resistant bacteria is progressing rapidly, endangering the efficacy of antibiotics and available therapeutic strategies, which leads to a growing interest in treatment methods using

natural compounds. Essential oils (EOs) and their constituents are well-known for their promising antimicrobial properties (Bassole and Juliani, 2012). Among them, *Cinnamomum zeylanicum* and *Cinnamomum cassia* bark essential oils contain up to 80% of *trans* cinnamaldehyde (*t*-CA) (3-phenyl-2-propenal) (Rao and Gan, 2014), which has previously

Abbreviations: AZM, azithromycin; CFU, colony forming units; DEGs, differentially expressed genes; EHEC, enterohemorrhagic *Escherichia coli*; EO, essential oils; GO, gene ontology; LEE, Locus of Enterocyte Effacement; mitC, mitomycin C; MBC, Minimum bactericidal concentration; MIC, Minimum inhibitory concentration; SHX, serine hydroxamate; *t*-CA, *t*-cinnamaldehyde.

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<https://doi.org/10.1016/j.phymed.2024.155845>

Received 7 February 2024; Received in revised form 14 June 2024; Accepted 25 June 2024

Available online 26 June 2024

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demonstrated antibacterial, antifungal, and anti-parasitic properties (Knauth et al., 2018). Recognizing its potent antimicrobial properties, *t*-CA mechanism of action has been the subject of intense research recently (Doyle and Stephens, 2019). Previous studies have shown *t*-CA and its derivatives significantly reduce biofilm formation by human pathogens. This activity is considered as a result of interfering with quorum sensing (QS) signaling, particularly through inhibiting *p*_{lux} promoters expression and autoinducer synthesis. *t*-CA was shown to affect cell division by inhibiting the expression of *ftsZ* and the FtsZ protein activity, by binding through the *t*-CA carbonyl group, leading to growth inhibition of Gram-negative and Gram-positive model strains (Doyle and Stephens, 2019). Other studies indicated the effect of *t*-CA on membrane potential and permeability, resulting in the oxidation of membrane proteins, cytoplasm leakage and acidification of the cytoplasm (Oussalah et al., 2006). These studies also demonstrated the effect of *t*-CA in reducing the toxicity of enterohemorrhagic *E. coli* (EHEC) (Oussalah et al., 2006). However, the exact mechanism of this activity has remained unknown. *t*-CA is approved for consumption by international agencies such as the European Food Safety Authority (EFSA) or Food and Drug Administration (FDA); its acute toxicity is considered relatively low (2 g/kg). The global intake of this natural propenal in the daily diet is solid evidence of its safety and thus it can be considered a promising agent towards infection control.

Expression of Shiga toxins is the main virulence factor of EHEC strains (Henrique et al., 2022). EHEC is a zoonotic pathogen listed among the 9 pathogens commonly transmitted by food, leading to periodic pandemics even in highly developed countries. The infections with EHEC mostly through contaminated food (where the main reservoir is cattle) lead to the hemorrhagic colitis with possible serious sequelae, which are related to one of the most important EHEC virulence factor, Shiga toxin. Despite its health burden, effective EHEC therapies remain elusive (Khalil et al., 2018). This is directly related to the mechanism of Shiga toxin synthesis. The enterotoxin is encoded in the genome of lambdoid bacteriophages, integrated with the bacterial chromosome (Henrique et al., 2022). The *stx* gene expression correlates with phage lytic development, activated by late phage promoters, causing toxin release post-cell lysis. Prophage activation occurs via factors inducing the SOS response following DNA damage (Glinkowska et al., 2010). Such factors include antibiotics, which, when used during EHEC infections, worsen patient conditions (Henrique et al., 2022). The most severe complication is hemolytic-uremic syndrome (HUS), associated with hemolytic anemia, acute renal failure, and thrombocytopenia. Children and seniors are at increased risk of HUS. Hence, there is an ongoing need to develop a new approach to control EHEC infections that allows reducing complications by inhibiting bacterial toxicity.

Earlier reports indicate the stringent response significantly controls EHEC virulence factor expression and lambdoid phage development (Nowicki et al., 2013). This global cellular reaction to environmental stresses like amino acid starvation alters gene expression (Potrykus and Cashel, 2008). This process is driven by specific nucleotides, tetra, and penta-phosphorylated guanosine collectively referred to as (p)ppGpp. In *E. coli*, their synthesis depends on two enzymes: the ribosome-associated RelA synthetase, which is activated during amino acid starvation when uncharged tRNA binds to the ribosome, and the SpoT synthetase, which is activated in response to other stress stimuli. Our findings have revealed that the induction of the stringent response may be a double-edged sword, leading to a reduction in the synthesis of bacterial enterotoxins in spite of being an adaptive mechanism to stress conditions (Krause et al., 2021; Nowicki et al., 2013). Namely, the RelA-dependent state of amino acid starvation results in inhibition of phage development which in turn leads to effective inhibition of Shiga toxin expression (Nowicki et al., 2016). This beneficial effect is evident for isothiocyanates (ITC), plant-derived compounds from the Brassicaceae family. The activity of sulforaphane, phenethyl isothiocyanate and other derivatives has had an inhibitory effect on bacterial growth combined with inhibition of EHEC toxicity.

The aim of our study was the evaluation of the *t*-CA activity against EHEC in terms of growth and Shiga toxin synthesis. We pursued the molecular mechanism of *t*-CA effect by metabolic, genetic, and transcriptomic approaches. We also performed *in vivo* evaluation of obtained data.

Methods

Antimicrobial activity and determination of bacterial growth inhibition

Most part of the study was conducted on *E. coli* MG1655, EHEC 933W, CB571, W4; all strains and their mutants used in this study are listed in Table 1. Bacterial cultures were typically grown in Mueller-Hinton (MH) medium (Sigma Aldrich, Darmstadt, Germany) or MH agar plates for all experimental procedures. The antibacterial activity of *t*-CA (97 %, Sigma Aldrich, C80687) was assessed by determining the minimum inhibitory concentration (MIC) following the CLSI M07-A9 guidelines as described previously (CLSI, 2012). In brief, inoculum was prepared using colony suspension (equivalent to a McFarland 0.5, in 3 ml of MH) from colonies grown within 18 to 20 hours. MIC was determined using a two-fold microdilution broth assay with 96-well plates, with concentrations ranging from 15 to 0.117 mM. The plates were then incubated at 37 °C overnight, and bacterial suspensions were plated on MHA plates to evaluate the minimum bactericidal concentration (MBC). The growth inhibition was determined spectrophotometrically (OD₆₀₀) using the EnSpire (Perkin Elmer Singapore Pte. Ltd., Singapore) microplate reader. Fresh bacterial cultures were shaken in the presence of appropriate *t*-CA concentrations in LB broth at 37 °C.

Time-kill kinetics

The bacterial inoculum with a standard optical density of 0.5 McFarland was diluted in LB broth to obtain a concentration of 5 × 10⁵ CFU/ml as previously reported (Klepser et al., 1998). The suspensions were then incubated with antimicrobial agents, and samples were

Table 1
Strains employed in this work.

<i>E. coli</i> strain	Relevant genotype or description	Reference
MG1655	F– λ– <i>ilvG rfb-50 rph</i>	(Jensen, 1993)
MG1655Δ <i>relA</i>	As MG1655 but <i>relA251::kan</i>	(Xiao et al., 1991)
MG1655Δ <i>relAspoT</i>	As MG1655 but <i>relA256spoT212</i>	(Nowicki et al., 2013)
MG1655(933W)	MG1655 lysogenic with 933W Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Nowicki et al., 2013)
MG1655(φ24B)	MG1655 lysogenic with φ24B Δ <i>stx</i> ₂ :: <i>cat</i>	(Nowicki et al., 2013)
MG1655(P27)	MG1655 lysogenic with P27 Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Nowicki et al., 2013)
MG1655Δ <i>ldcC</i>	MG1655 Δ <i>ldcC</i> :: <i>kan</i>	Lab collection
PT22	PT22 lysogenic with P22 Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Gamage et al., 2004)
PT27	PT27 lysogenic with P27 Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Gamage et al., 2004)
PT32	PT27 lysogenic with P32 Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Gamage et al., 2004)
PT38a	PT 38a lysogenic with P38a Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Gamage et al., 2004)
PT38b	PT38b lysogenic with P38b Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Gamage et al., 2004)
PT39a	PT39a lysogenic with P39a Δ <i>stx</i> ₂ :: <i>catGFP</i>	(Gamage et al., 2004)
86–24Δ <i>tox</i>	O157:H7 lysogenic with ST2–8624 Δ <i>stx</i> :: <i>GFP</i> instead of wild-type phage	(Loś et al., 2008)
CB571	O157:H7 <i>stx1</i> , <i>stx2</i>	(Nowicki et al., 2016)
EDL933W	O157:H7 <i>stx1</i> , <i>stx2</i>	(Nowicki et al., 2016)
W4	O157:H7 <i>stx1</i> , <i>stx2</i>	Lab collection

collected over time, serially diluted in PBS buffer, and plated on agar plates for colony counting after overnight incubation at 37 °C. The action of compounds was deciphered according to established criteria: >99.9 % CFU/ml reduction for bactericidal and <99.9 % CFU/ml for bacteriostatic action. Data from three independent experiments were presented as log₁₀ curves.

Bactericidal effect assessment by bioluminescence approach

In the experiment, *Vibrio harveyi* BB7 strain was utilized as a luminescence indicator (Srinivasan et al., 2017). Bacteria were inoculated in LBS broth at 30 °C with shaking and cultured for 2 h. Subsequently, the cultures were centrifuged, washed twice, and suspended in fresh LBS broth (HCl-Tris pH 7.5, Brain-heart infusion, NaCl 3 %). Bacterial suspensions were then placed in white-walled 96-well plates containing the tested antibiotics, and luminescence measurements were taken over time using the EnSpire microplate reader. The results (expressed in relative luminescence units, RLU) from three independent experiments were normalized to the bacterial cell density (OD₆₀₀) and presented as mean values with standard deviation (SD).

Evaluation of reactive oxygen species (ROS) levels

Intracellular ROS induction was assessed similarly as described previously (Nowicki et al., 2016). Bacteria were cultured in MOPS broth to achieve OD₆₀₀ = 0.5. After triple washing of the cells with PBS, the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma Aldrich) was added to a final concentration of 30 µM, and the mixture was incubated with shaking at 37 °C in the dark for one hour. The bacteria were then centrifuged and washed twice with PBS, followed by approximately 10-fold concentration in PBS. Hydrogen peroxide (1 µM) or *t*-CA at concentrations ranging from 0.25 to 2 × MIC was added. The prepared suspensions were incubated for 2 h at 37 °C. Fluorescence signals were measured using a fluorescence spectrophotometer with an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Lysine decarboxylase assay

The lysine decarboxylase (CadA protein) activity was quantified as presented previously (Phan et al., 1982). Briefly, overnight cultures of wild-type MG1655 and Δ ldcC isogenic strain were diluted 1:100 in LB broth and grown until reaching an OD₆₀₀ of 0.1. Then 10 ml of cultures were treated with subinhibitory concentrations of *t*-CA (0.125–0.25 × MIC) or exposed to pH 5.5 (adjusted with acetic acid). Untreated culture was used as a control. After 60 min of exposure, 1 ml of samples were harvested 4000 x g, 10 min, resuspended in buffer A (1 M NaCl, 0.05 K₂HPO₄ pH 6.5) and chilled on ice. Cells were centrifuged (4000 x g, 10 min) and resuspended in 200 µl of phosphate buffer (20 mM KH₂PO₄, pH 6.5). Cell free extract was obtained by chloroform extraction and vortexing by 30 s and stored on ice. Subsequently, 50 µl of cell extract was moved to pre-warmed (37 °C) reaction buffer (5 mM L-lysine, 0.1 mM pyridoxal phosphate, K₂HPO₄ pH 6.5). Reaction was stopped after 15 min with 120 µl 1 M Na₂CO₃ and chilled on ice. 120 µl of 10 mM 2,4,6-trinitrobenzenesulfonic acid (TNBS) was added to each sample and developed in 1 ml of toluene. The CadA activity expressed in cadaverine, the by-product of lysine decarboxylation, concentration was analyzed spectrophotometrically A₃₄₀ as N,N'-bistrinitrophenylcadaverine is soluble in toluene. The experiment was performed in three independent biological replicates and results were normalized to untreated control.

Evaluation of intracellular nucleic acids synthesis efficiency during the *t*-CA treatment

The synthesis of nucleic acids was evaluated by measuring the incorporation of radioactive precursors, [³H] thymidine, and [³H]

uridine (for DNA and RNA, respectively) (Hartmann Analytics GmbH, Braunschweig, Germany) based on a well-established procedure (Nowicki et al., 2016). Overnight bacterial cultures were diluted 1:100 in LB broth and grown until reaching an OD₆₀₀ of 0.1. Then, 5 µCi of [³H] thymidine or [³H] uridine was added. Samples of 50 µl were collected over time and placed on Whatman No. 3 filter paper. The filters were quickly transferred to ice-cold 10 % trichloroacetic acid (TCA) for 10 min, followed by 5 % TCA and then twice to 96 % ethanol. After a series of washes, the filters were dried, and radioactivity was measured using a MicroBeta scintillation counter (PerkinElmer, Downers Grove, IL, USA). The results (expressed in counts per minute, cpm) from three independent experiments were normalized to the bacterial cell density (OD₆₀₀) and presented as mean values with standard deviation (SD).

Assessment of (p)ppGpp alarmone levels

The levels of (p)ppGpp were measured as described previously with slight modifications (Nowicki et al., 2016). Bacteria cultured on LA plates were suspended in minimal MOPS medium with low phosphate concentration (0.4 mM) to an turbidity of 0.5 McFarland. They were then cultured with the addition of orthophosphoric acid [³³P] (1 µCi/ml) (Hartmann Analytics GmbH) for two generations. At time zero, *t*-CA was added at a concentration of 1 x MIC or 0.5 x MIC, and serine hydroxamate (SHX) at a concentration of 3 mg/ml was used as a positive control of stringent response induction. Samples were collected at indicated time (15, 30, 60 min) and subjected to lysis with formic acid (13 N) and then frozen and thawed twice. After centrifugation (5 min at 14,000 x g), nucleotides were separated using thin-layer chromatography on PEI-cellulose plates in 1.5 M potassium phosphate buffer (pH 3.4). Chromatograms were analyzed using the Phosphorimager Typhoon 9200 (GE Healthcare, Sweden). QuantityOne software was used for densitometric analysis.

Phage lytic development assay

The evaluation of prophage induction efficiency and lytic development was carried out as follows: Overnight cultures of the relevant lysogenic *E. coli* strains (933 W and Φ24B) were diluted 1:100 in LB broth and shaken at 37 °C until reaching OD₆₀₀ of 0.1. Then, mitomycin C (1 µg/ml), *t*-CA at a concentration of 1 x MIC or 0.5 x MIC, or a combination of both was added to the cultures. An untreated control sample was also included. Samples of 100 µl were collected over time, and 100 µl of chloroform was added to each sample. The mixture was vortexed, and then supernatants were serially diluted in TM buffer (10 mM Tris, 10 mM MgSO₄, pH 7.2). Two-microliter drops of the diluted supernatants were spotted onto Taq nutrient agar plates mixed with the indicator strain *E. coli* MG1655. The bottom agar was supplemented with 2.5 µg/ml chloramphenicol, as described previously (Łoś et al., 2008). The experiment was repeated three times, and the results were presented as mean values with standard deviations (SD).

Evaluation of *t*-CA cytotoxicity and efficacy in the treatment of EHEC infections using *G. mellonella* model

Larvae of the greater wax moth *G. mellonella* (Lepidoptera: Pyralidae) purchased from Exoticroom, (Łódź, Poland) were stored in the dark at 20 °C prior to use (Budziaszek et al., 2023). For all experiments, just healthy larvae weighing about 250–300 mg without signs of melanization and devoid of black spots in the cuticle were used. First, to exclude the toxicity of *t*-CA towards the larvae, various concentrations of the *t*-CA ranging from 12.5 mg to 100 mg per kg of body weight, were administered to the last left proleg by injection. The larvae were kept then at 37 °C for 10 days and observed daily for signs of acute response. For the assessment of treatment efficacy, the insects were infected with bacteria at a concentration of 10⁸ per larva using a Hamilton syringe with a blunt-ended needle into the last left proleg. After one hour of

infection, therapeutic compounds such as azithromycin (6.25 mg per kg of b.w.) and/or *t*-CA (50 mg per kg of b.w.) were administered to the insects in the last right proleg. Control larvae were injected with PBS. The larvae were placed on Petri dishes with Whatman filter paper and incubated in darkness at 37 °C. Observations were conducted every 24 h for the following five days, monitoring changes in behavior and melanization. Death was determined by the lack of movement and dark pigmentation of the larvae's epidermis. The procedures were repeated at least three times with $n = 10$ per group, the Kaplan–Meier survival curves was prepared based on one representative experiment.

Cell membrane permeability test

The *E. coli* MG1655 and EDL933W strains were cultured as described in the previous section. Subsequently, cells were incubated with *t*-CA or mitomycin C for at least 2 h. Untreated samples were used as controls. To assess the impact of *t*-CA on cell membranes, 10 µM propidium iodide (PI) was added to the bacteria and incubated in darkness for 10 min. The cells were immobilized on thin pieces of 1 % agarose placed on glass slides. Images were captured using a Leica DMI4000B microscope equipped with a DFC365FX camera (Leica) and N2.1 filter. The images were collected and processed using LAS AF 3.1 software (Leica).

Evaluation of Shiga toxin expression

The expression of the Shiga toxin was monitored using a reporter gene fusion of the *stx* gene and GFP protein (Gamage et al., 2004). The lysogenic strains of bacteria (*E. coli* 933 W Δ *stx*:GFP and MG1655 Δ *relA* Δ *stx*:GFP) were cultured in the same manner as in the phage induction experiment. Microscopic analysis was conducted as described in the previous section, using *t*-CA, mitomycin C, and SHX as controls. Visualization of fluorescence signal of expression from *p*_{stx} promoter was performed using a green fluorescent protein (GFP) filter. This approach allowed to evaluate the GFP signal in the hemolymph of *G. mellonella* larvae after infection with *E. coli* 8624 Δ *stx*:GFP. Larvae ($n = 5$) were treated with *t*-CA and azithromycin or mixture. Hemolymph was collected using a pipette after making a small incision in the larva's abdomen with a sterile scalpel and gently pressing on the insect until a drop of hemolymph appeared. The drop was collected into Eppendorf-type tubes containing an anticoagulant buffer. Hemolymph was spread onto microscopic slides and visualized in a fluorescence microscope as described above.

Assessment of bacterial morphology with Transmission electron microscopy (TEM)

TEM was employed to investigate structural changes in cell morphology subsequent to *t*-CA treatment. Bacteria were cultured under standard conditions in LB medium. The MIC of *t*-CA was added to bacterial cultures (OD₆₀₀ = 0.4) and incubated in 37 °C for 3 h. Nontreated cultures were used as control at the designated time point, the bacteria were collected by centrifugation (4000 × g, 10 min), washed twice with PBS and resuspended in a fresh medium. Afterward, 5 µl of the diluted (100 times) bacteria culture was dropped and adsorbed onto carbon-coated grids (GF Microsystems), directly stained with 1.5 % uranyl acetate (Sigma-Aldrich) and visualized with an electron microscope. The analysis was conducted using the Tecnai G2 Spirit BioTWIN TEM (FEI Company, Eindhoven, Netherlands) at 120 kV.

RNA extraction and sequencing

Briefly, freshly prepared overnight culture of the MG1655 strain was recultivated (1:100) in LB medium at 37 °C with continuous shaking until an optical density at 600 nm (OD₆₀₀) of 0.1 was obtained. Following this, *t*-CA was introduced at a concentration equivalent to 0.25 × MIC. Control cultures were left untreated. At designated time

intervals (30, 60 min), 1 ml of each culture was harvested and mixed with 5 % AquaFenol in 96 % ethyl alcohol. Subsequently, the samples were promptly cryopreserved in liquid nitrogen and stored at -20 °C. For RNA isolation, the frozen samples were first thawed on ice and then centrifuged at 4000 × g for 5 min. The resulting cell pellet was resuspended in 100 µl of Tris-EDTA (TE) buffer (10 mM Tris-Cl; 1 mM EDTA; pH 8.0) supplemented with lysozyme (1 mg/ml) and incubated at room temperature for 10 min. Subsequent purification steps were carried out employing the RNase Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. To ensure thorough removal of DNA, DNase I (Qiagen) treatment was implemented during the column-based purification process. The elution of RNA was performed in 50 µl of RNase-free water, and the isolated RNA samples were stored at -80 °C until further analysis. The quality and integrity of the isolated RNA were meticulously assessed employing the Agilent 2100 Bioanalyzer system in conjunction with the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA), in accordance to the manufacturer's guidelines. Transcriptome sequencing on the NovaSeq6000 platform was performed by MacroGen Europe.

RT-qPCR analysis

Total RNA was isolated from bacterial samples in procedure described for RNA-seq and reversely transcribed with a hexamer primer and SuperScript™ III First-Strand Synthesis SuperMix for RT-qPCR (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The resulting cDNA was then used as the template for qPCR. Primers used in this study and reaction conditions are listed in table S1. Samples were assayed in triplicate and PCR reactions without the template were performed as negative controls. The comparative Ct method for relative quantification ($\Delta\Delta$ Ct method) was used to analyze the data.

Next-generation sequencing data analysis

Raw data in Fastq format underwent quality control of reads using FastQC. The data was uploaded to the Galaxy platform, utilizing the public server located at usegalaxy.org (The Galaxy Community, 2022). Subsequently, the reads were mapped to the reference genome of *E. coli* str. K-12 substr. MG1655 (GCA_000005845), obtained from Ensembl Bacteria release 57 – July 2023 (Martin et al., 2023). Based on the mapped sequences, read counts for annotated genes were tallied using the FeatureCounts function. To identify differential gene expression, the DESeq function was employed. All these analyses were conducted within the Rstudio R-4.3.1 environment. Genes exhibiting a 1.5-fold change between the treated and control samples with a significance level of $p < 0.001$ were further proceeded in DAVID (The Database for Annotation, Visualization and Integrated Discovery) platform for Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses (Sherman et al., 2022). Additionally, the associations between significantly altered genes were explored utilizing the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) platform version 12.0.

Data availability

Transcriptomic data from this work can be found at Gene Expression Omnibus under accession number GSE252441.

Statistics and data analysis

Experiments were conducted at least three times and statistical analyses were performed with Prism version 8.4.3 (GraphPad Software, Inc.) using Student's *t*-test, *G. mellonella* survival curves were plotted using Kaplan-Meier method and estimated using long-rank Mantel-Cox test. Experimental significance was defined as $p < 0.05$ for differences between reported mean values.

Results

Sensitivity of *E. coli* strains to *t*-CA treatment

First, we performed an evaluation of the sensitivity of *E. coli* strains to *t*-CA treatment to determine its activity (Fig. 1). The two-fold microdilution method was employed for evaluating minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The MIC and MBC were in range 1.875 or 3.750 mM and 3.75 or 7.50 mM respectively (depending on the strain tested, Fig. 1B, Table S2). Importantly, the EHEC were more sensitive to *t*-CA than the wild-type K12 derivative strain. Then, we verified whether *t*-CA's action exhibits bacteriostatic or bactericidal properties on pathogenic strains used in our study. EHEC members, namely EDL933W, W4, CB571 response to *t*-CA treatment was compared with kanamycin and tetracycline in time-killing assay (Fig. 1C). The *t*-CA effectivity against isolates was comparable to observed for MG1655 wild type *E. coli*. As indicated on Fig. 1C the log³ reduction in cell counts was obtained for 1 x MIC of *t*-CA after 3 h of incubation, revealing the bactericidal activity of the compound. Moreover, even a subinhibitory concentration of 0.5 x MIC *t*-CA was able to kill 99.9 % cells of EDL93W and CB571 strains at 4 h post exposure. To validate these findings, a bioluminescence assay was conducted employing the *Vibrio harveyi* BB7 strain as it was shown as a valuable method of antibiotic compound effectiveness evaluation (Ortiz de Garcia et al., 2016). Obtained results were in line with those in time-kill assay assessed for *E. coli*. Namely, it was found that *t*-CA shows activity compared to bacteriostatic and bactericidal antibiotics, as significant reduction in relative light units (RLU) was observed after 25 min following the addition of agent to the culture (Fig. 1D). The effect of *t*-CA was most like the one observed for tetracycline in respect to the time-frame of RLU reduction (Fig. 1D).

t-CA affects cell morphology and membrane permeability

The observed growth inhibition can be attributed to various effects of the compound. To determine the detailed bacterial structure after the phytoncide treatment, TEM technique was employed (Fig. 2A, Fig. S1). Both the wild-type *E. coli* and the O157:H7 EDL933W strain displayed cell deformation characterized by cell contraction and detachment of the cell membrane from the wall. Density of cytoplasm in treated cells was also affected with observed condensation. Untreated bacteria exhibited a normal morphology. Fluorescence analysis of PI indicated the presence of a bacterial population with increased membrane permeability, both following *t*-CA and mitomycin C (mitC) treatment (where mitC was used as a positive control for induction of prophage excision in lysogenic strains). Moreover, induction of the bacteriophage lytic cycle led to distinct changes in cell's shape, resulting in its filamentation. Consequently, cell membrane disintegration and bacterial death occurred. Meanwhile, untreated cells showed no dye fluorescence (Fig. 2B).

t-CA treatment results in broad modulation of gene expression

Given the antibacterial potential of *t*-CA and its effectiveness against EHEC, we aimed to investigate whether the observed physiological changes could be tracked at the level of gene expression. To gain insight into potential molecular pathways involved in *t*-CA antibacterial activity, we performed transcriptomics analyses of *E. coli* MG1655 cells exposed to subinhibitory concentration (0.94 mM, equivalent to 0.25 x MIC) comparing to untreated cells, t = 30 and 60 min. This approach aimed to capture the initial signs of the compound activity and direct the focus toward crucial cellular targets. We observed potent modulation of gene expression (Fig. 3, Dataset 1). Following treatment with 0.25 x MIC, we found 292 and 140 upregulated and 107 and 96 downregulated genes at time points 30 and 60 min, respectively (Fig. 3A and Dataset 1)

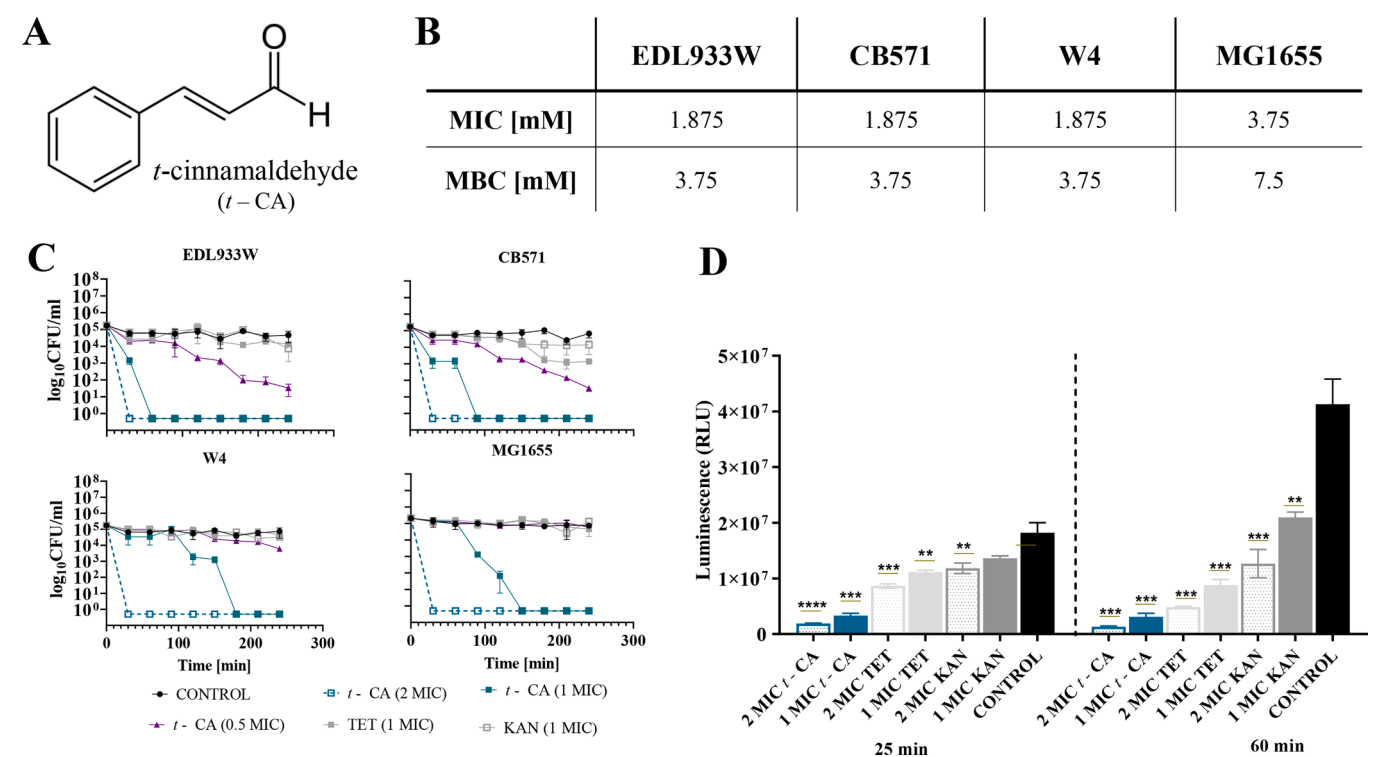


Fig. 1. Analysis of bacterial susceptibility to *trans*-cinnamaldehyde (*t*-CA) treatment. (a) *t*-CA structure scheme (b) Inhibitory and bactericidal (MIC, MBC) concentrations determination for *E. coli* MG1655 and enterohemorrhagic (EHEC) strains (EDL933W, W4, CB571); results shown as median of three independent replicates in triplicate. (c) Comparative killing-curves analysis of *t*-CA, tetracycline, and kanamycin on EHEC (d) Luminescence intensity (RLU) evaluation of *V. harveyi* after exposure to *t*-CA or antibiotics at indicated concentrations (compared with blank control (black bar), **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

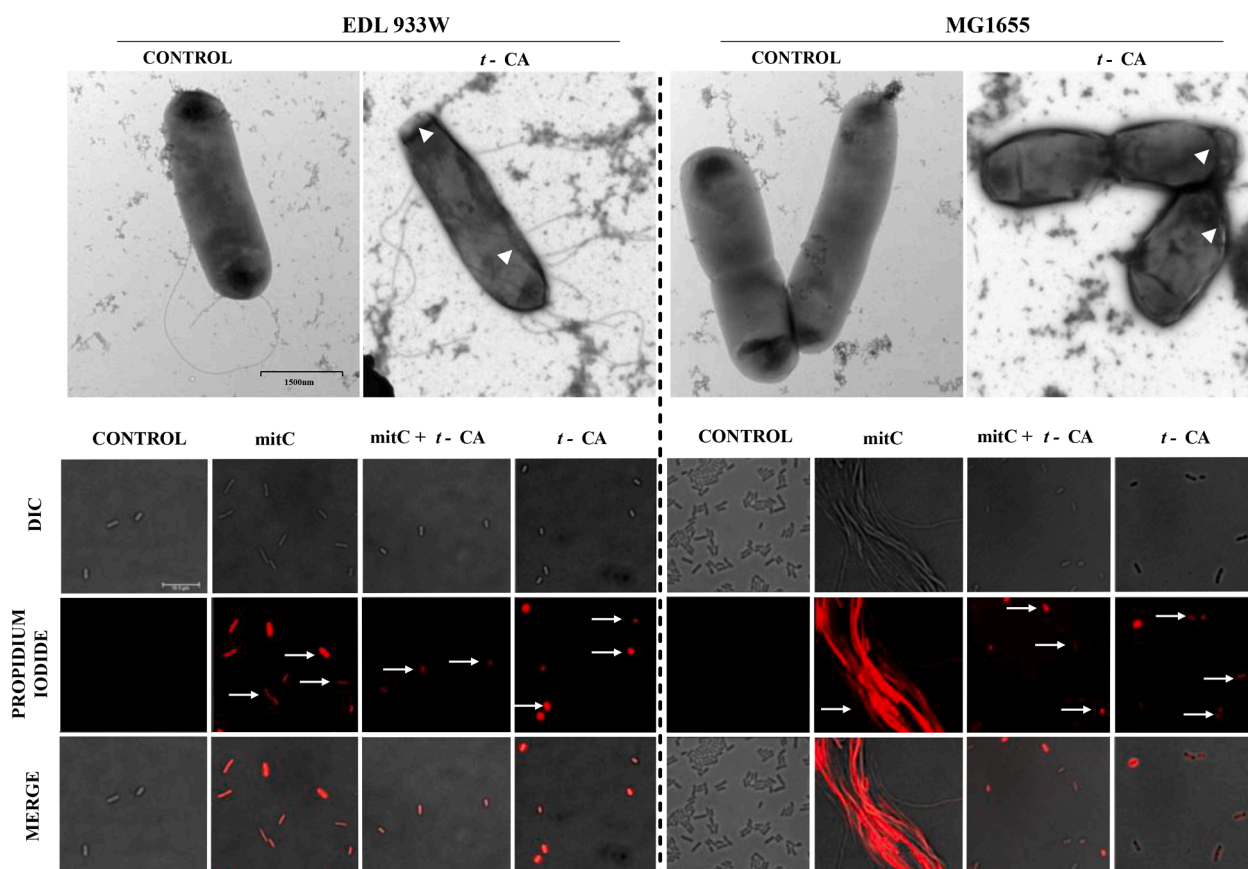


Fig. 2. Morphological and physiological impact of *trans*-cinnamaldehyde (*t*-CA). Transmission Electron Microscope (TEM) images showing EDL933W and wild-type MG1655 strain structures post 1x MIC *t*-CA treatment, highlighting detached cell membranes from the cell wall (white arrowheads). (b) Fluorescence microscopy images of EDL933W and wild-type MG1655 cells stained with propidium iodide (PI) after treatment with 1x MIC *t*-CA. Mitomycin C (mitC, 1 mg/ml) was used as a positive control. The cells with increased permeability were marked by white arrows. At least 100 cells were screened for a specific condition, representative micro-images are presented.

(fold change ≥ 3 or ≤ -3 , $p < 0.001$). The diversity of Differentially Expressed Genes (DEGs) has been presented in a Venn diagram and demonstrates the 136 overlapping genes whose expression is increased or decreased at both time points (Fig. 3B). Clustering between the groups is shown by principal component analysis (PCA) (Fig. 3C) and indicates within-group consistency.

Subsequent analysis of DEGs using the DAVID analysis tool identified 16 functional clusters (Fig. 3D, Dataset 2), highlighting significant involvement in the tricarboxylic acid (TCA) cycle, flagellum synthesis, amino acid transport, and oxidoreductase activity, based on Enrichment score (Es) (Dataset 3). Notably, the transport cluster was the largest, with 75 DEGs, followed by oxidoreductase activity and transcription regulation with 50 and 43 DEGs respectively. Fifteen DEGs related to amino acid transport specifically encoded transmembrane proteins for amino acids like Ala, Arg, Thr, Met, Cys, Glu, Gly. The oxidoreductase function cluster included genes related to electron transport, NADH synthesis, and cytochrome. On the other hand, DEGs in the transcription regulation category were most abundantly represented by transcripts encoding repressors or factors associated with negative regulation of this process (such as *araC*, *trpR*, *torR* or *soxR*).

Further analysis using the STRING database v12.0 (Szklarczyk et al., 2019) revealed a network of gene interactions, as shown in Fig. 4A. In this approach, DEGs were collated from both time points (pooled list), and then analyzed $|\geq 1.5|$ $\log_2$ FC changes for selected pathways. This way, we were able to focus on the most significantly altered processes. Notably, genes involved in cellular metabolism, electron transport, as well as a cluster of flagellum-related genes, turned out to be the predominant groups (Fig. 4A). The observed dynamics of transcriptional

changes in metabolic processes after *t*-CA treatment indicated a down-regulation of transcripts in the pathways of glycolysis/gluconeogenesis, pyruvate, and fatty acid metabolism; however, with some exceptions associated with *poxB*, *cadA* or *tor* and *nar* operons where upregulation was observed (Fig. S2, Dataset 1). Changes in the expression of selected genes from the indicated pathways was then validated by RT-qPCR (Fig. 4B) and were in line with transcriptomic data. We also confirmed the activity of the *cadA* gene product, encoding an inducible lysine decarboxylase enzyme, in colorimetric L-lysine degradation assay (Fig. 4C) using a strain deficient in constitutive lysine decarboxylase LdcC. These overexpressed genes are considered as triggers of catabolic processes or supporters of anaerobic growth. In particular, *tor* operon genes are known to be involved in the bet-hedging strategy (Carey et al., 2018), in response to changing environmental conditions. Namely, *torR*, *torC* and *torA* were significantly upregulated in our analyses (5.7, 6.0, 3.9 $\log_2$ FC, respectively at 60 min). Another group of genes significantly upregulated in response to *t*-CA treatment were those involved in the nitrate reduction (*narVWYZ*). The overexpression of genes associated with activation of alternative respiratory pathways may also indicate energy deficits that the cell tries to overcome. Taken together, these findings suggest a pattern of transcriptome changes consistent with coping with cellular stress conditions.

Deciphering the mechanism of stress response and prophage induction

The stringent response, known as a crucial global stress response that regulates metabolic changes but also influences bacteriophage development, was investigated in relation to *t*-CA's effects on nucleic acid

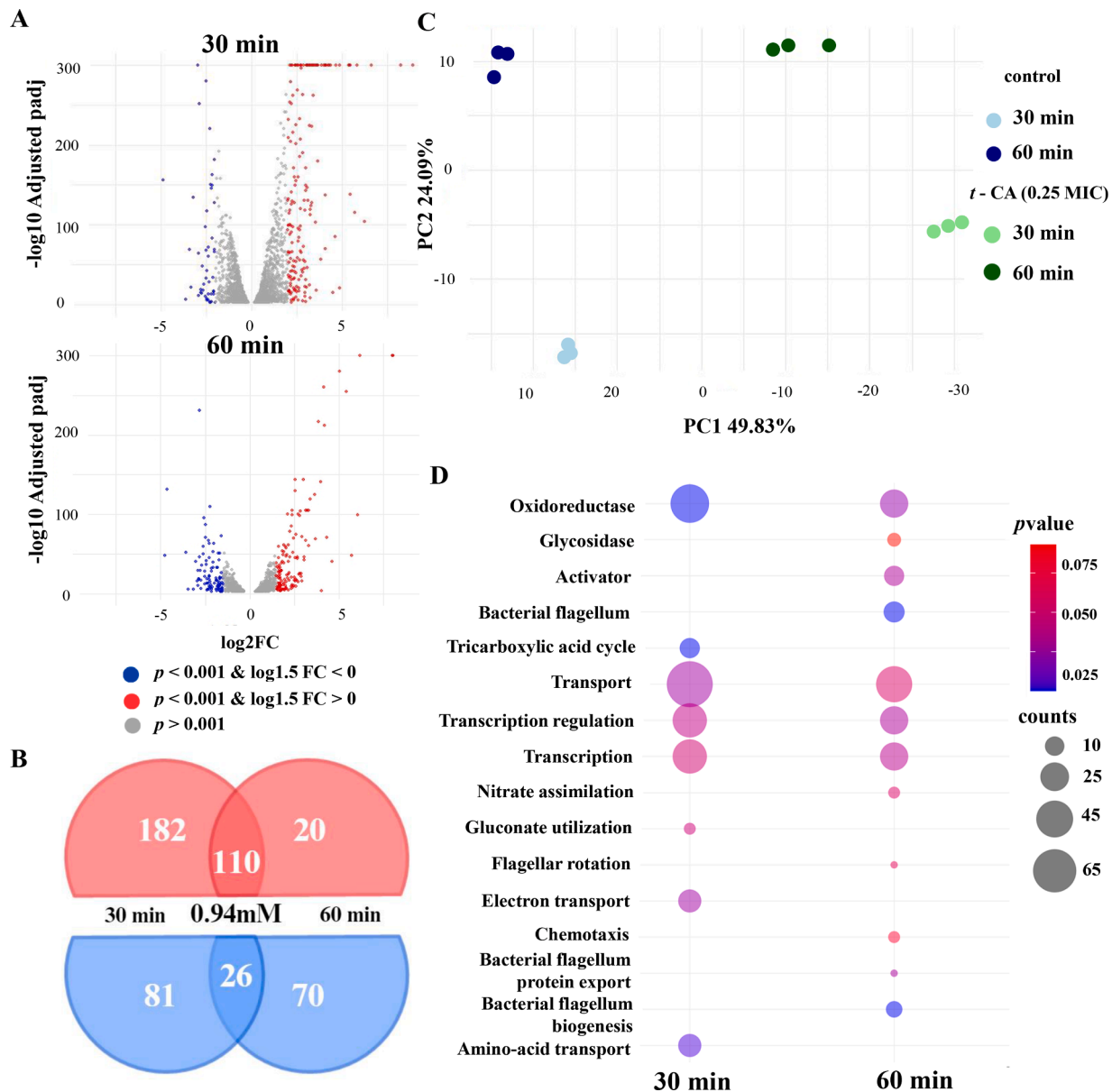


Fig. 3. Transcriptomic changes upon *trans*-cinnamaldehyde (*t*-CA) treatment. Differentially expressed genes (DEGs) analysis upon treatment with 0.25 x MIC *t*-CA was performed against untreated control in indicated time with $p \leq 0.001$ and $\log_2FC \geq 1.5$ threshold and visualized by R software with ggplot2 package. (a) Volcano plots of DEGs. Red dots mean up-regulated genes, blue dots mean down-regulated genes, and gray dots mean no difference genes. (b) Venn diagram showing overlapping DEGs among groups. (c) Principal component analysis (PCA) of transcriptome data of treated and untreated cells at indicated times (d) Gene Ontology (GO) Functional enrichment of DEGs according to DAVID Functional annotation clustering.

synthesis as a measure of stringency induction (Fig. 5A, B). Under stringent conditions, the transcriptional capacity of RNA polymerase are altered, leading to stable RNA down-regulation and significant disruption of DNA replication due to growth arrest (Potrykus and Cashel, 2008). Both DNA and RNA synthesis were found to be rapidly inhibited by *t*-CA in wild-type cells ($p < 0.05$) (Fig. 5A, B), while no such inhibition was observed in mutant strains with deletions in *relA* and *relA spoT*, suggesting the effects seen in wild-type cells are dependent on the alarmones of the stringent response. However, we were still able to observe the growth inhibition of tested mutant strains (Fig. S3). To further confirm the role of *t*-CA in inducing the stringent response, levels of (p)ppGpp, a key stringent response alarmone, were measured (Fig. 5C, D). Significant accumulation of (p)ppGpp was induced by *t*-CA in wild-type *E. coli*, comparable to that induced by serine hydroxamate (SHX), a known stringent response inducer ($p < 0.05$). This accumulation was found to be dose-dependent (Fig. 5D). However, in $\Delta relA$ strain,

the presence of (p)ppGpp was not detected following treatment with either *t*-CA or SHX, indicating that the induction of the stringent response by *t*-CA is mediated through the (p)ppGpp synthesis pathway controlled by RelA.

ROS generation, phage induction, and toxin expression

Next, given that prophage induction plays a significant role in EHEC virulence, we investigated whether *t*-CA, could catalyze the generation of ROS a potent trigger factor of SOS response. We utilized 2,7-dichloro-fluorescein diacetate (DCFH-DA), a fluorescent probe indicative of ROS in cells. Hydrogen peroxide was employed as a positive control. We tested a range of active *t*-CA concentrations from 0.25 to 2 x MIC. In *t*-CA the treated samples, ROS levels were reduced compared to the untreated control. (Fig. 6A). Furthermore, the levels of natural ROS donors, namely NAD/NADH decreased over time with increasing doses of *t*-CA

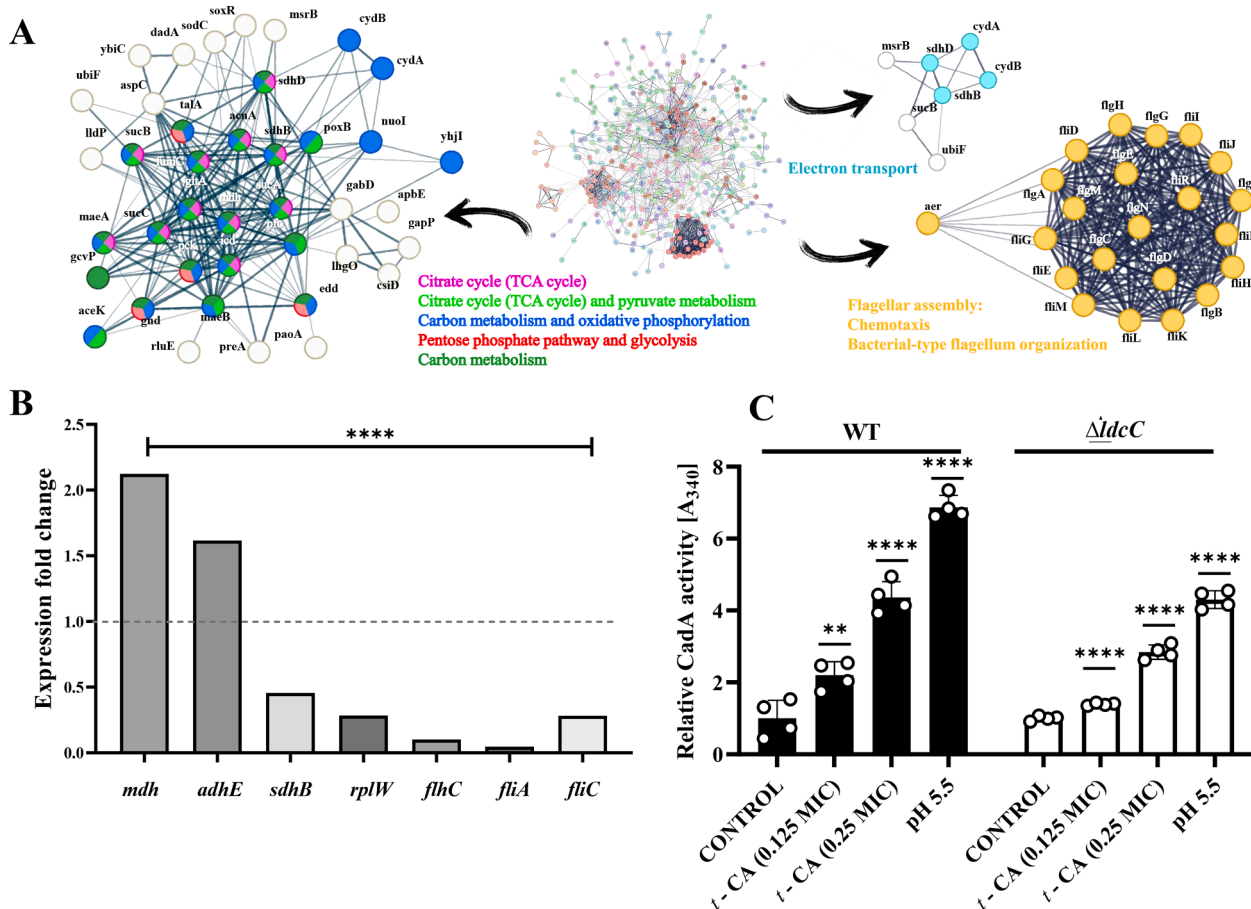


Fig. 4. The effects of *trans*-cinnamaldehyde (*t*-CA) on metabolic pathways and general processes. Differentially expressed analysis of 0.25 x MIC *t*-CA was performed against untreated control at indicated time with $p \leq 0.001$ and $\log_2FC \geq 1.5$. (a) STRING functional network clustering for pooled differentially expressed genes (DEGs) from both time groups. Markov cluster (MCL) analysis with inflation parameter 2 was used to visualize the interactions. (b) The set of genes involved in changed cellular processes were examined using RT-qPCR analysis in EDL9333W strain (c) Quantitative analysis of CadA lysine decarboxylase activity expressed as a relative value to untreated cells (black bar, ** $p < 0.01$, *** $p < 0.001$).

(Fig. S4).

Then, we also checked whether the action of *t*-CA leads to the activation of the phage lytic development. We employed 3 lysogenic strains harboring Stx bacteriophages, namely 933 W, $\Phi 27$, and $\Phi 22$, to determine whether the phytochemical can induce their excision and assembly in bacteria. We observed that the development of lambda-like phages was inhibited in the presence of 0.5 and 1 x MIC *t*-CA, even under the treatment of a strong inducer like mitC. A distinct pattern emerged when using prophage-bearing strains with deletions in the *relA* gene, suggesting that a defect in stringent response alarmones synthesis contributes to the effective action of *t*-CA (Fig. 6B). The results were found to be consistent for the tested strains underscoring the universality of the effect.

To assess toxin gene expression, we utilized the MG1655 933W strain, which incorporates green fluorescent protein (GFP) in place of the Shiga toxin genes, allowing for the visualization of promoter activity through fluorescence microscopy (Fig. 6C, Fig. S5AB). Cells subjected to mitC presented characteristic filamentous shape of intracellular lytic phage development, accompanied by robust GFP expression. In contrast, *t*-CA -treated cells lacked cellular elongation in microscopic images, accompanied by the absence of GFP signals. Consistently, these effects were reversed in *relA* strains (Fig. 6C, Fig. S5B). Hence, our conclusion was that *t*-CA effectively impairs both, phage lytic development and *stx* gene expression in bacterial cells.

Suppression of Shiga toxin expression and in vivo validation

Finally, we sought to determine if the observed *in vitro* anti-virulence effect of the *t*-CA translates into the inhibition of infection symptoms in an *in vivo* animal model using *G. mellonella* larvae (Fig. 7). The effectiveness of the larval model has been well-established, making it an optimal surrogate for infection studies and the testing of new antibacterial compounds (Champion et al., 2016). Prior to conducting the infection model studies, we assessed the safety of *t*-CA administration to larvae. We applied a range of *t*-CA concentrations from 12.5 to 100 mg/kg b.w. Our observations suggest that none of the tested concentrations had a toxic impact on the *G. mellonella* model, as we did not observe melanization symptoms associated with worsened insect condition (data not shown). We also established the bacterial dose of EDL933W for larval survival in the experiment, which was 10^7 cells per larva (Fig. S6). As a positive control for experiments, we selected azithromycin (AZM). AZM exhibits activity against most common diarrheagenic pathogens, such as *E. coli*, *Shigella* spp., *Salmonella* spp., or *Campylobacter* spp., and is used to combat specific Enterobacteriaceae (Denham et al., 2018). Growth kinetic observations of EDL933W bacteria indicated that AZM and *t*-CA in combination inhibited bacterial growth more effectively than separately (Fig. S7B), exhibiting significant additive effects in checkerboard assay (Fig. S7A). The insects were then administrated with bacterial load and received treatment one hour after infection (Fig. 7B). The inclusion of *t*-CA as a therapeutic agent (50 mg/kg b.w.) in the *G. mellonella* model significantly increased the

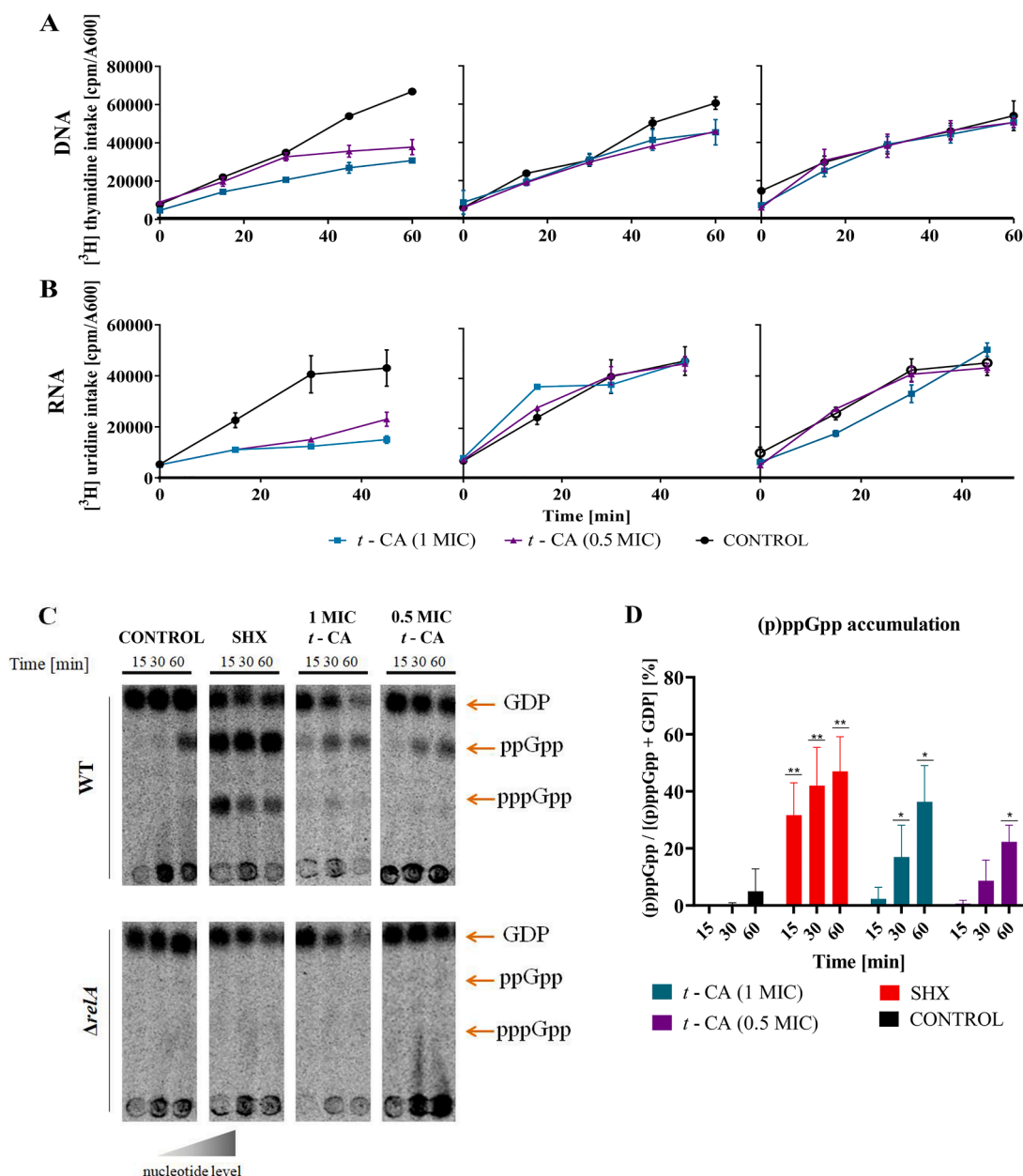


Fig. 5. *Trans*-cinnamaldehyde (*t*-CA)-induced alterations in DNA/RNA synthesis and stringent response in *E. coli* (a–b) DNA and RNA synthesis in wild-type *E. coli* strains, $\Delta relA$, and $\Delta relA \Delta spoT$ (ppGpp⁰) in the absence (black circles) or presence of *t*-CA at 1 x MIC (turquoise) and 0.5 x MIC (purple). Results represent mean values from three independent experiments conducted in duplicate, with error bars indicating SD. (c) Synthesis of stringent response alarmones, ppGpp and pppGpp, assessed by [³³P] labelling of nucleotides in wild-type or *relA*, and *relA spoT* deficient bacteria with serine hydroxamate as a positive control. The positions of ppGpp and pppGpp on the TLC plates are indicated by arrows. (d) Relative synthesis of alarmones (** $p < 0.01$, *** $p < 0.001$).

survival rate compared to control organisms treated with PBS ($p < 0.05$). The observed effectiveness of *t*-CA was comparable to that of AZM (6.25 mg/kg b.w.) (Fig. 7B).

Furthermore, larval survival following AZM combined with *t*-CA administration was significantly higher than monotherapy. Then, utilizing the similarities in the larval model's immune system with higher animal models, we decided to investigate the response to virulence expression *in situ*. In this approach, we employed O157:H7 strain, which encodes GFP instead of the Shiga toxin gene to infection experiments. This allowed us to assess the impact of *t*-CA and its combination with AZM on Shiga toxin expression in larvae hemolymph. Under microscopic examination, hemocytes in the control group without treatment exhibited a strong GFP protein fluorescence signal. In trials involving *t*-CA and AZM, GFP expression was diminished (Fig. 7C, Fig. S7C).

Discussion

t-CA, a longstanding phytochemical in traditional medicine, has proven efficacy against various pathogens (He et al., 2019; Pereira et al., 2021; Shen et al., 2015). We present here data confirming *t*-CA's bactericidal efficiency against *E. coli* in concentrations of 1.80–3.75 mM, aligning with previous reports (Fig. 1, Table S2). However, till now, the background of observed antibacterial mechanisms of *t*-CA has remained unclear.

The effect of *t*-CA on the integrity of the cellular membrane has already been reported in various bacteria, including *E. coli* and leading to the increased permeability (He et al., 2019). Initial evidence was cytoplasmic shrinkage in *t*-CA-exposed cells, indicating starvation state (Fig. 2A, Fig. S1) (Shi et al., 2021). Our research solidifies these findings

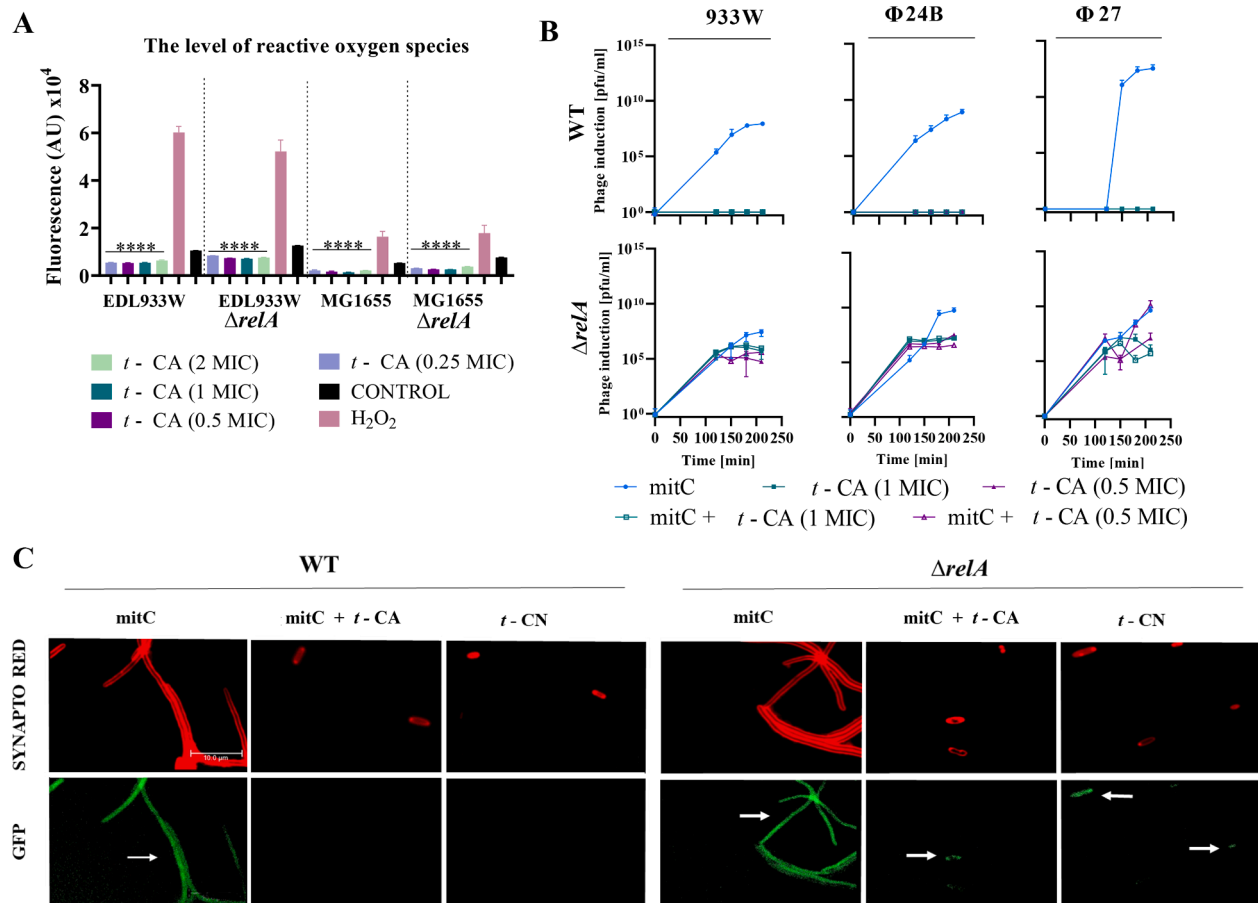


Fig. 6. Trans-cinnamaldehyde (t -CA) impact on phage induction, lytic development and *stx* gene expression (a) Levels of reactive oxygen species (ROS) in the wild-type MG1655 and EDL933W strains and their *relA* mutants after treatment with t -CA at 0.5, 1 $\times$ MIC. (b) Phage yield efficiency in lysogenic strains of MG1655 and its *relA* mutants treated with t -CA. mitC was used as the inducing agent (positive control). Results from three replicates were normalized to culture density, and the phage yield from autoinduction was subtracted. (c,d) Lysogenic phage 933W $\Delta stx2::GFP$ was induced by mitC (1 μ g/ml) in the presence or absence of t -CA or SHX, where GFP fluorescence corresponds to the expression of *stx* genes (indicated by arrows). Induction was assessed for wild-type *E. coli* strains and $\Delta relA$ mutants. Untreated cells were used as controls.

by revealing substantial membrane damage following t -CA treatment (Fig. 2B). To investigate the molecular basis of t -CA antibacterial properties, we assessed its effect on the metabolic processes and stress response.

The global effect of t -CA has been assessed by the transcriptomic approach which revealed alterations in several crucial pathways, notably observed at subinhibitory concentrations of the drug. Significantly down-regulated genes included those in fatty acid metabolism, hinting at causes of membrane structure deterioration, of which fatty acids are a major constituent (Shi et al., 2021). Processes associated with cellular energy, particularly glycolysis and the TCA cycle were mostly down-regulated. This was in line with data presented by Visvalingam et al (Visvalingam et al., 2013). However, the picture that emerged from our approach indicates a redirection of metabolism to the alternative pathway characteristic to the stationary phase of growth, e.g. the *poxB* gene, whose transcription was among the most upregulated in our analysis. Notably, PoxB is involved in pyruvate metabolism during the transition from exponential to stationary growth (Bernal et al., 2016). One of the by-products of pyruvate metabolism is acetate, thus, elevated PoxB activity leads to significant acidification of the cytoplasm. This phenomenon of t -CA action was previously linked with membrane disruption (Doyle and Stephens, 2019). Interestingly, we observed strong overexpression of the *cadA* gene, responsible for lysine degradation at low pH. We confirmed enhanced CadA activity in t -CA treated cells (Fig 4C). The upregulation of CadA by t -CA activity under

conditions of deregulated metabolism and impaired transportation machinery leads to lysine depletion in cells resulting in a strong starvation signal. Thus, these data indicate the global stress conditions caused by t -CA.

The stress produced by t -CA consequently induces the stringent response which could be both the effect and the cause of the observed changes in gene expression and anti-virulence mechanism. Moreover, the promoters of lambdaoid bacteriophages are subject to strong regulation by stress alarmones (Nowicki et al., 2013). The important part of studying any antimicrobial compound against EHEC is the threat to induce the *stx*-bearing prophages and the production of Shiga toxin, as many antibiotics do through producing SOS response. Our results show that t -CA is able to counteract the genotoxicity of quinolone, mitomycin C, as shown for three distinct Stx phage strains (Fig. 6B) due to RelA activity. Furthermore, no increase in cellular ROS or potential radical donors like NADH was observed (Fig. 6A, S4). In addition, the RNA-seq analysis conducted here revealed that expression of genes involved in the SOS response such as *sulA*, *dinB*, *recA*, *ruvA*, *sbmC*, *umuD*, *yebG*, and *lexA* were not significantly altered compared to the control (Dataset 1). It implies that t -CA activity is not associated with provoking the SOS response orchestrated by these factors. The accumulation of the alarmone (p)ppGpp (Fig. 5) shifted cellular machinery into energy-saving mode, strongly inhibiting flagellum assembly, a change we also observed (Sun et al., 2022) observed also by us (Fig. 4, Fig. S2). Moreover, the previously observed effect of t -CA on cell division via FtsZ

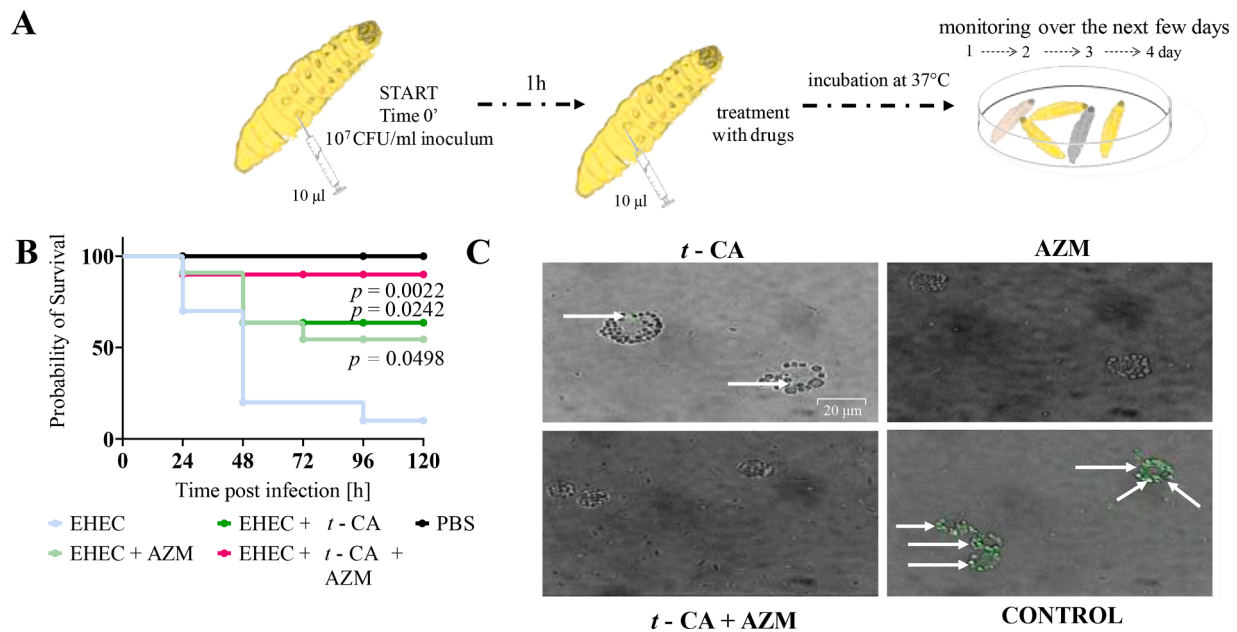


Fig. 7. Effects of *trans*-cinnamaldehyde (*t*-CA) on enterohemorrhagic *E. coli* (EHEC) infection in *G. mellonella* (a) Schematic pipeline of *in vivo* studies using *G. mellonella* larvae. (b) Kaplan-Meier survival curves of *G. mellonella* after infection with 10^7 cells/larva of EDL933W strain and treatment with azithromycin (AZM), *t*-CA, and combination (AZM + *t*-CA). The EHEC infected group was used as a control for statistical analysis (c) Representative micrographs of *G. mellonella* hemocytes after infection with enterohemorrhagic strain 8624 Δ stx2::GFP and treatment with *t*-CA or AZM or *t*-CA/AZM. Larvae not subjected to treatment were used as controls. Expression of GFP (Shiga toxin) is indicated by arrows.

(Doyle and Stephens, 2019) could also be a consequence of alarmone activities, as both FtsZ protein expression and polymerization are strictly regulated by (p)ppGpp (Yamaguchi et al., 2015).

To assess the therapeutic potential of *t*-CA against EHEC we employ a surrogate infection model of *G. mellonella* (Champion et al., 2016). Our findings are consistent with other studies demonstrating *t*-CA's antibacterial efficacy *in vivo* against pathogenic bacteria (Upadhyay and Venkitanarayanan, 2016). Surprisingly, Kaplan-Meier analysis showed *t*-CA effectiveness similar to AZM's ($p < 0.05$), with 60 % and 50 % survival rates at 96 h a.i. Moreover, *t*-CA facilitated the cellular response and immune defense mechanisms in *G. mellonella*, as observed by a substantial decrease in virulence linked to the toxin production in hemocytes extracted from infected larvae that had undergone treatment. These data mark an initial step towards *t*-CA's potential use in EHEC therapy.

The most important point to reveal in our study was what is a mechanism of *t*-CA-mediated induction of the stringent response and subsequent virulence inhibition of EHEC strains. The major effect on central metabolism pathway, especially genes encoding the enzymes of

TCA cycle, exerted by *t*-CA, together with the alteration of pyruvate metabolism, as illustrated by elevated *poxB* transcript level, led us to propose the following model. *t*-CA provides the signal for the alternative metabolic pathways, including carbon metabolism and affecting the important player connecting several pathways, pyruvate. The elevated PoxB level provokes acid stress for the cell. This *per se* could be a signal for the induction of the stringent response, however here additional pathway plays an important role. Notably, the cell has a mechanism to protect against the acid stress, utilizing the lysine decarboxylase CadA to form cadaverine. Indeed, the *cadA* transcript was among the most up-regulated upon *t*-CA treatment. The increased CadA activity deprives the cell of lysine, as we observed by increased cellular cadaverine levels upon *t*-CA treatment (Fig. 4C). The lysine limitation induces then the stringent response activating RelA enzyme. The alarmone, (p)ppGpp, affects bacterial metabolism leading to growth inhibition and down-regulates the prophage lytic promoters activity, and by such, the *stx* gene expression (Fig. 8). This multistep effect of *t*-CA provides an elegant model for anti-EHEC activity.

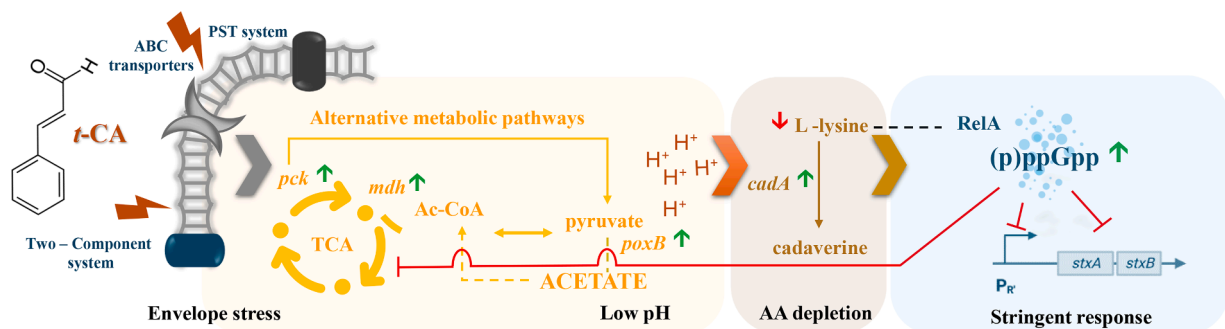


Fig. 8. Schematic representation of anti-EHEC virulence targeting of *trans*-cinnamaldehyde (*t*-CA). Proposed mechanism *t*-CA provides the signal for the alternative metabolic pathways, affecting the important player connecting several pathways, pyruvate. Elevated PoxB levels provokes acid stress in cells, triggering CadA-mediated L-lysine decarboxylation. This amino acid depletion activates the ribosome-associated RelA factor, leading to overproduction of the alarmone (p)ppGpp. (p)ppGpp specifically inhibits phage lytic promoters and enhances metabolism redirection.

Conclusions

In summary, our research highlights *t*-CA therapeutic potential against EHEC isolates, by the bacterial growth inhibition and, more importantly, by targeting Shiga toxin synthesis by prophage excision attenuation. The mediator of this effect is the stringent response alarmone, (p)ppGpp, its induction caused by the *t*-CA-triggered metabolic redirection for alternative pathways, affecting pyruvate metabolism and indirectly causing lysine limitation. This mechanism allows for rapid growth inhibition of bacteria paralleling the decrease in their virulence, as shown in larvae model, especially when *t*-CA effect is combined with azithromycin. Thus, further investigations of potential interactions between *t*-CA and other antibacterial agents can facilitate the development of more comprehensive therapeutic strategies.

CRediT authorship contribution statement

Monika Karczewska: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Ai Yan Wang:** Investigation. **Magdalena Narajczyk:** Investigation. **Bartosz Słomiński:** Methodology. **Agnieszka Szalewska-Pałasz:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Dariusz Nowicki:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Science Center SONATA grant no. UMO-2018/31/D/NZ7/02258.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.phymed.2024.155845](https://doi.org/10.1016/j.phymed.2024.155845).

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Article

Increased Levels of (p)ppGpp Correlate with Virulence and Biofilm Formation, but Not with Growth, in Strains of Uropathogenic *Escherichia coli*

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Abstract: Urinary tract infections are one of the most frequent bacterial diseases worldwide. UPECs are the most prominent group of bacterial strains among pathogens responsible for prompting such infections. As a group, these extra-intestinal infection-causing bacteria have developed specific features that allow them to sustain and develop in their inhabited niche of the urinary tract. In this study, we examined 118 UPEC isolates to determine their genetic background and antibiotic resistance. Moreover, we investigated correlations of these characteristics with the ability to form biofilm and to induce a general stress response. We showed that this strain collection expressed unique UPEC attributes, with the highest representation of FimH, SitA, Aer, and Sfa factors (100%, 92.5%, 75%, and 70%, respectively). According to CRA (Congo red agar) analysis, the strains particularly predisposed to biofilm formation represented 32.5% of the isolates. Those biofilm forming strains presented a significant ability to accumulate multi-resistance traits. Most notably, these strains presented a puzzling metabolic phenotype—they showed elevated basal levels of (p)ppGpp in the planktonic phase and simultaneously exhibited a shorter generation time when compared to non-biofilm-forming strains. Moreover, our virulence analysis showed these phenotypes to be crucial for the development of severe infections in the *Galleria mellonella* model.

Keywords: UPEC; ppGpp; stringent response; virulence; UTI; biofilm; *E. coli*; *Galleria mellonella*; phenotype; pathogenicity; infection; antibiotic resistance



Citation: Karczewska, M.; Strzelecki, P.; Bogucka, K.; Potrykus, K.; Szalewska-Pałasz, A.; Nowicki, D. Increased Levels of (p)ppGpp Correlate with Virulence and Biofilm Formation, but Not with Growth, in Strains of Uropathogenic *Escherichia coli*. *Int. J. Mol. Sci.* **2023**, *24*, 3315. <https://doi.org/10.3390/ijms24043315>

Academic Editors: Giuseppe Zanotti and Jintae Lee

Received: 21 December 2022

Revised: 26 January 2023

Accepted: 6 February 2023

Published: 7 February 2023



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1. Introduction

Urinary tract infections (UTIs) are among the most frequently occurring major bacterial infections worldwide, and their significant increase in incidence in recent years is alarming [1–3]. It is estimated that 150 million UTIs occur annually worldwide, with significant morbidity and high treatment costs affecting the efficiency of national health care systems [4]. In the US alone, each year, more than 10 million doctor office visits, 2 million emergency department visits, and 100,000 hospitalizations are associated with UTIs [5,6]. Those at high risk are newborns, pre-school girls, sexually active women, and older people of both sexes [7]. UTIs mainly affect the female population, which is related to the structure of the urinary tract. A cohort study among 2000 women representing the US population corroborates this finding [8,9].

The prevalence of UTIs results in rampant use of antibiotics, which impacts the spread of resistance. This is a clinical problem, especially in recurrent UTIs [10]. Therefore, empirical treatment of emergency room patients is a challenge for clinical urology and requires the constant evaluation of bacterial susceptibility to antibiotics

UTIs have been associated with such bacterial species as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, and those belonging to the Enterobacteriaceae family, such as *Klebsiella pneumoniae*, *Proteus mirabilis*, *Citrobacter*, and *Enterobacter*, as well as some *Candida* species [11,12]. However, uropathogenic *Escherichia coli* (UPEC) are most commonly responsible for about 80% of uncomplicated UTIs, 95% of out-of-hospital infections, and 50% of hospital-acquired infections [13]. UPEC are included in the ExPEC pathotype, along with other *E. coli* isolates involved in various extraintestinal infections. UPEC strains have evolutionarily adapted to colonize a niche in the urinary tract. They harbor genetic virulence factors (VF) on pathogenicity-associated islands (PAIs) [14], contributing to bacterial pathogenesis and distinguishing them from commensal strains [15]. Phylogenetic analyses have shown that *E. coli* strains classify into four major phylogenetic groups (A, B1, B2, and D), of which those belonging to groups B2 or D are most often responsible for extraintestinal infections [16]. Eight phylogenetic groups are currently recognized by the classification established by Clermont: seven (A, B1, B2, C, D, E, F) belong to *E. coli sensu stricto*, while a distinct example is *Escherichia cryptic clade I* [17].

UPEC virulence requires coordinated expression of multiple genes that facilitate adhesion and colonization in the urinary tract. One can distinguish between VFs bound to the bacterial cell surface and those that are secreted and exported to the site of action. Adhesion to tissues within the urinary tract is the most important step in pathogenicity and is enabled by adhesion factors. These include various types of fimbriae (types 1, P, S, F1C), fimbrial Dr adhesins, as well as afimbrial Afa adhesins and PapC. Among the toxins that help bacteria spread to deeper tissues after disrupting cell integrity and that can cause an inflammatory response, the most important are the lipoproteins— α -hemolysin (HlyA) and cytotoxic necrosis factor 1 (CNF1) [18,19]. Moreover, the main component of the cell wall—lipopolysaccharide (LPS), antigen O and K—is an endotoxin that helps suppress the host immune system [20]. The availability of iron is very limited in the urinary tract, so in order to survive in this environment, bacteria encode proteins of iron-acquiring systems. UPEC strains are particularly well-equipped with the factors required for biosynthesis and uptake of the following siderophores: enterobactin, aerobactin, yersiniabactin and salmochelin, which they then transport into their cytoplasm through the Sit system (siderophore-iron transporter proteins, SitABCD) [21].

Biofilms are increasingly recognized as major contributors to pathogenicity, recurrence, chronicity, and recalcitrance-to-treatment of UTIs, as well as many other diseases. Biofilm is defined as a structured formation of bacterial cells adhering to a surface, surrounded by a polymeric matrix that they have produced [22]. Biofilm formation can be considered to be another virulence factor for UPEC strains, which promotes their survival in the urinary tract by protecting against the cleansing effects of hydrodynamic forces, host defense mechanisms, and antibiotics [23]. Currently, the ability to form a biofilm is not well understood, and several factors are generally considered to be involved in its formation, including adhesins and specific proteins such as antigen 43 [24]. Thus, the problem of frequent UTI recurrences and the tendency for UTIs to develop into a chronic form has been associated with biofilm formation during infection [25].

There is a general consensus in the research that the so-called stringent response is involved in the control of the pathogenicity of various bacterial species as a mechanism of bacterial adaptation towards changing environmental conditions through the modulation of gene expression. It is involved in processes related to growth, stress, starvation, and survival, and for many bacterial species, the stringent response is required for efficient expression of the virulence factors [26–28]. In the broadest terms, the stringent response is the global response of a cell to stress conditions resulting in inhibition of most processes requiring energy consumption [26]. The key molecules in the stringent response are the small-nucleotide alarmones, ppGpp, and pppGpp (guanosine tetra- and pentaphosphates) collectively referred to as (p)ppGpp. These alarmones are synthesized in *E. coli* by two enzymes. One is RelA, which responds to amino acid starvation, and the other is the SpoT enzyme, which is responsible for the synthesis of (p)ppGpp during other types of

stresses and deprivation, but additionally is involved in the hydrolysis of (p)ppGpp [26,29]. However, the importance of (p)ppGpp in the exponential growth rate control has also been presented [30]. The (p)ppGpp signaling controls global metabolic changes in response to environmental fluctuations and regulates such processes as biofilm formation, quorum sensing, adaptive processes, or bacterial virulence [31]. The entrance of cells into the stationary phase of growth is correlated with increased accumulation of intracellular (p)ppGpp [26]. This is caused by environmental constraints on growth, such as the depletion of nutrients or excess intermediate metabolites secreted into the environment, and it activates the effector proteins RelA and SpoT to synthesize the alarmones. This leads to changes in the expression of a number of metabolism-related genes, as well as the activation of the regulator FimB, which controls the phase variation of type 1 fimbriae in *E. coli* [32,33]. Type I fimbriae mediate the adhesion and invasion of UPEC and also are an important factor at the initial stages of biofilm formation [9,34,35].

Due to the prevalence of UTIs and their potential for recurrence and subsequent medical complications, UPEC infections are a serious health problem worldwide. The main aim of this study was to examine the genetic diversity of UPEC isolates from UTI patients from northern Poland. Furthermore, we conducted a phylogenetic clustering based on Clermont's classification, as well as testing evaluating susceptibility to basic antibiotics. In our thorough approach, we also investigated the UPEC strains' biofilm-forming ability (assessed by differentiation on Congo red agar), and evaluated their metabolic phenotypes by measuring the stringent response alarmones' basal levels, as well as assessing the growth rates of selected strains. Then, strains showing specific phenotypes were subjected to virulence analysis in a surrogate infection model of *Galleria mellonella*.

2. Results

2.1. Distribution of Virulence Factors and Phylogenetic Clustering of Isolates

First, we decided to evaluate the genetic background of the tested isolates, employing PCR analysis with previously published primer pairs regarding the above described virulence factors (VFs). According to the results of our genetic analysis, a relatively high distribution of VFs among the isolates studied (Figure 1a) is an important phenotypic characteristic, as one or more VFs were expressed in 100% of the strains. All of them encoded the *fimH* gene, and among the other factors analyzed, the most frequently identified were *sitA* (92.5%), *aer* (75%), and *sfa* (70%). The ability to express HlyA and/or PapC proteins, as well as antigen 43 occurred at a medium level (42.5–56.7%). In contrast, the lowest distribution was obtained for the *cnf*, *ibeA*, and *iss* genes (24.2%, 19.2%, and 18.3%, respectively). For 117 of the 120 strains, phylogroups were determined by extended quadruplex PCR. Of the 8 phylogroups described [17], 6 were identified in our collection (Figure 1b). A large number of isolates was assigned to group B2 (71.7%), followed by groups B1 (10.8%) and D (7.5%). UPEC isolates from groups F, A, and C were less frequently represented (3.3%, 2.5%, and 1.7%, respectively).



Figure 1. Genetic characteristics of the UPEC collection. (a) Percentage distribution of virulence factors identified by PCR in UPEC isolates. (b) Percentage distribution of phylogroups determined by phylogenetic typing using the quadruplex PCR method [17].

Based on the findings above, a series of analyses were carried out to determine the co-occurrence of virulence factors in relation to each other. Correlations were statistically confirmed with Fisher's exact test (Figure 2). In particular, many correlations were determined for the *sfa*, *papC*, and *agn43* genes. The least correlated with other VFs were the *ibeA*, *sitA*, and *aer* genes (Figure 2). For each pair, the direction of correlation was determined to be either positive or negative, using Pearson's test (Figure 2). A strong correlation was observed for the presence of the *cnf* gene, which was always present with the *hlyA* gene, while the presence of *hlyA* was not dependent on the presence of *cnf* (See supplementary material for details).

Fisher's exact test and Pearson's correlation

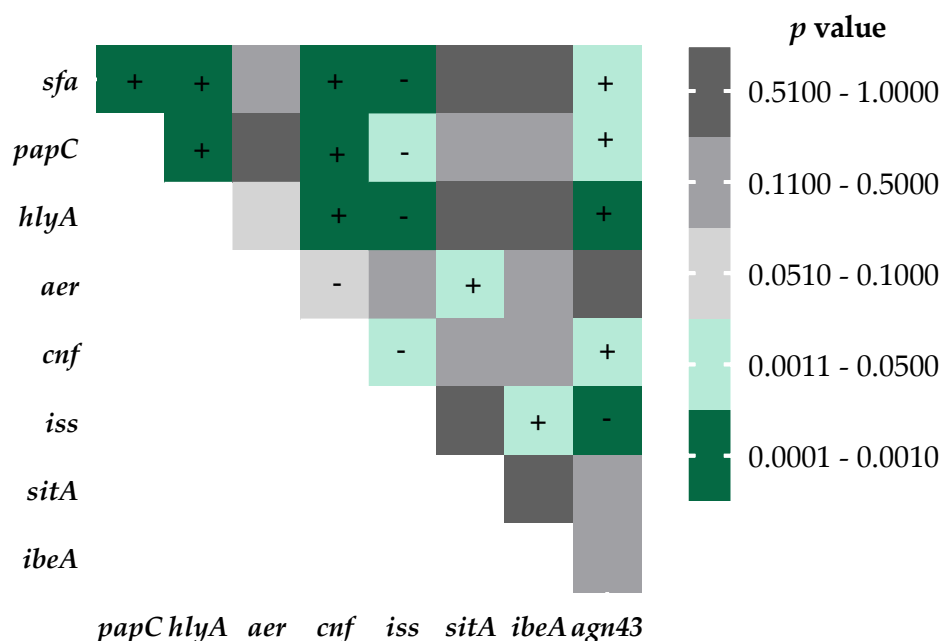


Figure 2. Co-occurrence matrix of virulence factors. Statistically significant results (Fisher's exact test, $p < 0.05$) are shown in green, while non-significant correlations are shown in gray. The direction of correlations was determined using Pearson's test, where "+" indicates a positive correlation and "-" a negative correlation ($p < 0.05$). The matrix presents horizontal values vs. vertical values.

Moreover, by including Clermont's classification in the analysis, we observed that different phylogenetic groups had different quantitative distributions of VF. The B2 group had the highest mean value (6.01), the A, C, and D groups had intermediate values, while B1 had the lowest value. Thus, the correlation between individual virulence factors and Clermont's phylogroups was analyzed. Some peculiarities should be noted, such as the occurrence of the *cnf* gene exclusively in the B2 phylogroup. Similarly, the *hlyA* or *ibeA* gene sequences are present only in this group, except for two isolates (Table 1). Due to the size of the groups, only the differences between the largest B1 and B2 groups were evaluated. Statistical analysis shows that these groups differ in the distribution of all analyzed genes except for *fimH*, *ibeA*, and *aer*, with the *fimH* sequence present in all strains, regardless of the group (Table 1).

Table 1. UPEC virulence genes categorized according to their occurrence in Clermont's phylogroups.

Virulence genes	No. (%) of UPEC Isolates Identified in the Clermont Phylogroups								Statistical Analysis of Distribution of Virulence Genes and Phylogenetics		
	A n = 3	B1 n = 13	B2 n = 86	C n = 2	D n = 9	F n = 4	Indefinite n = 3	Total n = 120	B1 ¹ n = 13	B2 ¹ n = 86	Fisher's Exact Test <i>p</i> Value (B1 vs. B2)
Adhesins											
<i>fimH</i>	3 (100)	13 (100)	86 (100)	2 (100)	9 (100)	4 (100)	3 (100)	120 (100)	ns	ns	ns
<i>sfa</i>	2 (67)	6 (46)	66 (77)	1 (50)	5 (56)	2 (50)	2 (66.7)	84 (70)	ns	0.0148	0.0398
<i>papC</i>	0	1 (8)	60 (70)	0	5 (56)	2 (50)	0	68 (56.7)	0.0002	<0.0001	<0.0001
Invasin											
<i>ibeA</i>	0	1 (8)	22 (26)	0	0	1 (25)	0	24 (20)	ns	0.0207	ns
Toxins											
<i>hlyA</i>	0	1 (8)	51 (59)	1 (50)	0	0	0	53 (41.2)	0.0061	<0.0001	0.0006
<i>cnf</i>	0	0	29 (34)	0	0	0	0	29 (24.2)	0.0364	<0.0001	0.0093
Iron Acquisition											
<i>aer</i>	2 (67)	9 (69)	63 (73)	2 (100)	7 (78)	4 (100)	3 (100)	90 (75)	ns	ns	ns
<i>sitA</i>	3 (100)	9 (69)	82 (99)	1 (50)	9 (100)	4 (100)	3 (100)	111 (92.5)	ns	ns	0.0097
Serum Resistance											
<i>iss</i>	2 (67)	6 (46)	10 (12)	1 (50)	1 (11)	0	2 (67)	22 (18.3)	0.0141	0.0042	0.0061
Biofilm formation											
<i>agn43a</i> <i>agn43b</i>	0	0	48 (55.8)	0	1 (11.1)	1 (25)	1 (33.3)	51 (42.5)	<0.0001	<0.0001	0.0001
Mean; Range of VF	4 (2–5)	3.52 (2–6)	6.01 (2–10)	4 (3–5)	4.11 (2–6)	4.25 (3–6)	4.66 (4–5)	5.43 (2–10)		<0.0001	

"ns" indicates that the result is not statistically significant. ¹ The statistical analysis of B1 and B2 individuals was conducted against the whole group of strains (n = 120).

The high genetic diversity of the strains is shown on a dendrogram (Figure 3). Based on the UPGMA method, a dendrogram was constructed in which 120 strains were grouped, based on the presence of virulence genes. Altogether, 51 different virulotypes were delineated. The most common genotypes were: (i) *fim*, *sfa*, *papC*, *hlyA*, *aer*, *sitA*, and *agn43*; (ii) *fim*, *sfa*, *papC*, *hlyA*, *aer*, *cnf*, *sitA*, and *agn43*; (iii) *fim*, *sfa*, *papC*, *aer*, and *sitA*; (iv) *fim*, *sfa*, *papC*, *cnf*, *sitA*, and *agn43*; and (v) *fim*, *sfa*, *cnf*, *sitA*, and *agn43*, and these accounted for 27.5% of the population studied. It is also worth noting that the most common virotypes present are characterized by a large number of virulence factors. Overall, the occurrence of three or more VFs was recorded in 115 isolates, which represents 95.8% of the studied population.

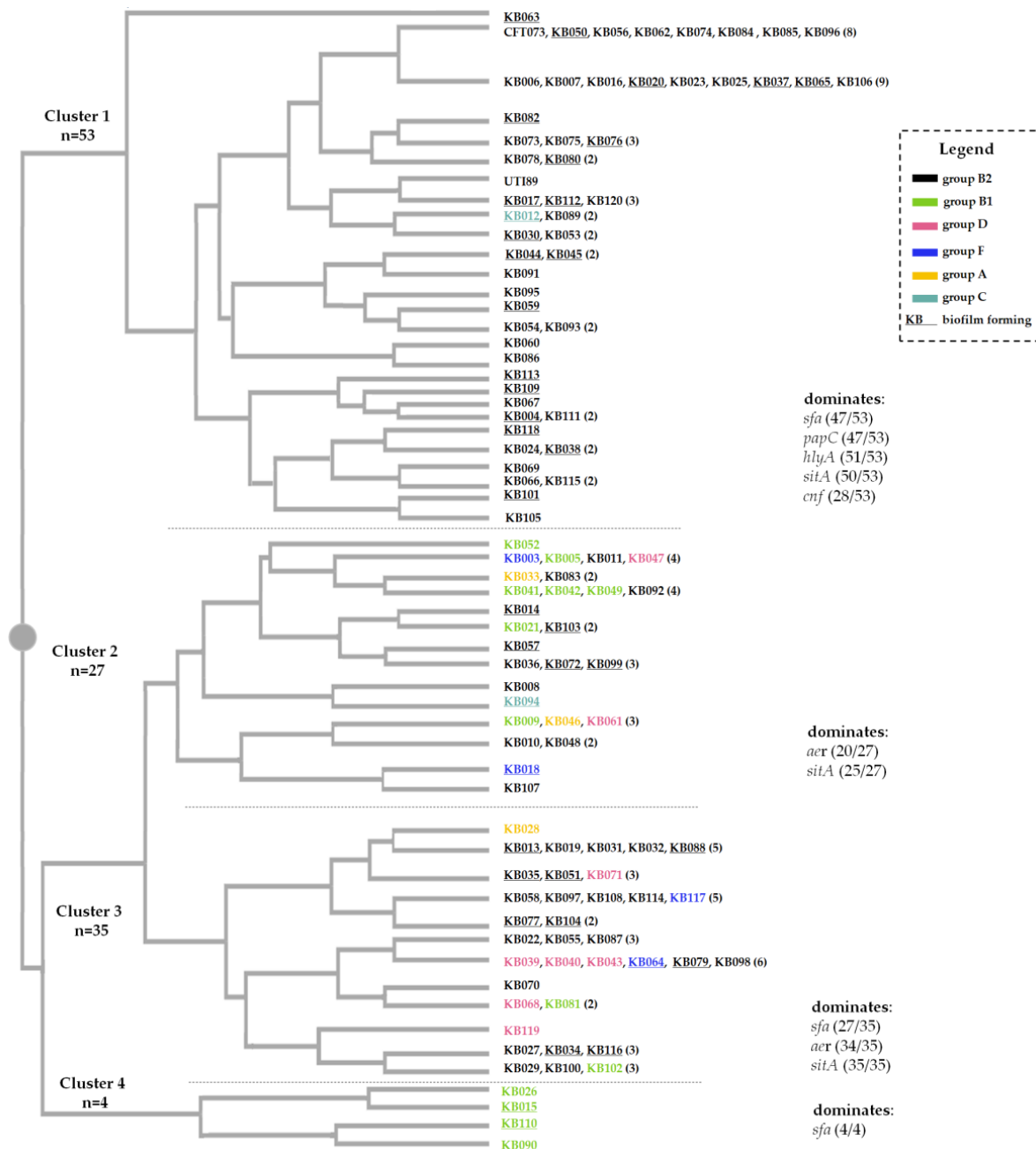


Figure 3. Genetic relationships among UPEC strains. The dendrogram was created by the UPGMA method using the DendroUPGAMA server [36]. Colors mark the different phylogenetic groups identified, and strains that form biofilm on Congo red agar are underlined. The dashed lines divide the dendrogram into four clusters. The first cluster brings together all strains encoding the *cnf* gene.

2.2. The Biofilm-Forming Phenotype Does Not Correlate with Genetic Background

Since the ability to form biofilm is an important pathogenicity factor, we decided to evaluate our collection according to this characteristic. In our collection, biofilm production on Congo red agar (CRA) was reported in 32.5% ($n = 39$) of UPEC. Intensely black colonies were present among 9 strains, 11 were moderately positive, and 19 were weakly positive (collectively referred to as CRA+). CRA+/- strains and phylogenetic groups are marked on the dendrogram (Figure 3). The link between biofilm formation and Clermont's phylogenetic classification was assessed. The phenotype of colonies on CRA (CRA+, CRA-) did not correlate with the phylogenetic groups, which was confirmed using Fisher's exact test (Table 2). In addition, whether this trait could be correlated with any of the virulence genes was also assessed. The analysis showed a significant co-occurrence of the IbeA factor in biofilm forming strains ($p = 0.0025$). No correlation was observed for other VFes.

Table 2. CRA biofilm formation ability in regard to UPEC phylogenetic groups.

Biofilm Formation by CRA	Phylogenetic Classification by Clermont						Fisher's Exact Test p Value
	A $n = 3$ Count (%)	B1 $n = 13$ Count (%)	B2 $n = 86$ Count (%)	C $n = 2$ Count (%)	D $n = 9$ Count (%)	F $n = 4$ Count (%)	
Biofilm forming ($n = 39$)	2 (66)	6 (46)	25 (29)	1 (50)	3 (33)	1 (25)	ns
Non-biofilm forming ($n = 81$)	1 (34)	7 (54)	61 (71)	1 (50)	6 (67)	3 (75)	

2.3. Biofilm Forming Abilities Correlate with a Multi-Resistant Phenotype

Since bacteria forming biofilms usually display multidrug resistance, we decided to assess this ability using our collection. In this study, we analyzed the susceptibility of UPEC strains to 16 antibiotics (Table S1). Resistance was noted in 53 strains (44.2% of the population), and among them almost half (52.8%) showed resistance to more than one antibiotic. Most strains displayed lack of sensitivity to ampicillin (92.5%). Chemotherapeutics (norfloxacin, ciprofloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole) also had a high resistance profile (58.5%). Of all the antibiotics evaluated, ceftazidime, meropenem, ceftibuten and fosfomycin were the most effective agents against the strains from our collection.

Whether the incidence of resistance was related to the age of the patient from whom the strain was isolated was also assessed. According to the phenotypic resistance evaluation, it was noted that the resistant strains (56.7%) were derived from individuals who were 50 years of age and older on the day of bacterial isolation. Moreover, these strains represented 71.7% of all resistant isolates in the collection. For the second group (below 50 years old), these percentages were 31.3% and 28.3%, respectively ($p = 0.0082$).

The relevance of the consequences of biofilm formation and drug resistance to the severity of the infection [37] prompted us to delve deeper into the characteristics of the strains and compare the different traits among them. We showed that the multidrug resistance profile of isolates was significantly more frequent in the group of strains capable of biofilm formation on CRA when compared to the non-biofilm forming group (Table 3).

Table 3. Biofilm-forming ability on CRA vs. the presence of a multiple resistance phenotype.

Group	Number of Resistant Strains (Multiple Resistant)	% of the Group (% of Multiple Resistance within Resistant Strains)	Fisher's Exact Test <i>p</i> Value
Biofilm forming n = 39	16 (12)	30.8% (75%)	0.0410
Non-biofilm forming n = 81	37 (16)	19.7% (43.2%)	

2.4. Higher Basal (p)ppGpp Level Results in Shorter Doubling Time in UPECs

It has been shown that (p)ppGpp levels negatively correlate with growth rate [30]. We wondered whether this would be a case here as well. Analysis of the basal level of the stringent response alarmone accumulation, (p)ppGpp, in the studied *E. coli* strains showed significant differences between the individuals strains. Some of them presented higher intrinsic levels of (p)ppGpp, and interestingly, this occurred under growth conditions without nutrient limitation. This trait turns out to be correlated with their ability to form biofilm on CRA, indicating a direct or indirect effect of (p)ppGpp on biofilm formation (Figure 4b). Another characteristic that differentiates groups of CRA+/- strains is the generation time (Figure 4b), with the CRA+ strains showing faster growth rates than the CRA- strains. The correlation of these two characteristics with the determined regression line is shown in Figure 4c.

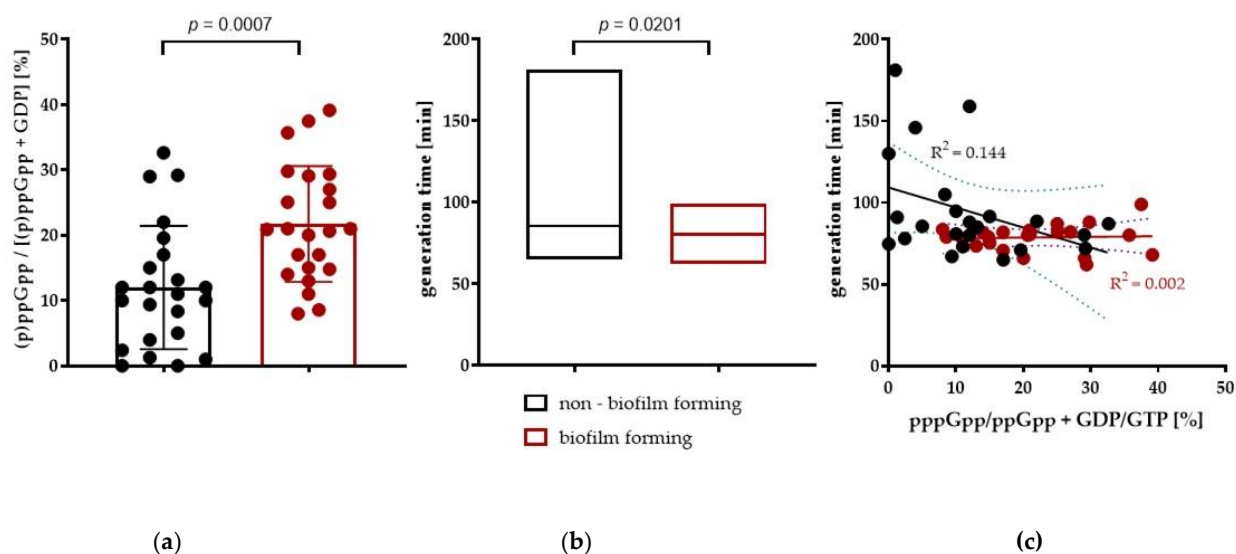


Figure 4. Phenotypic evaluation of the UPEC strains. Non-biofilm forming (n = 24) and biofilm-forming (n = 23) strains were grown aerobically in MOPS minimal medium (0.2% glucose), with shaking. (a) The relative accumulation of the (p)ppGpp alarmones was assessed by the ^{32}P labeling of nucleotides and revealed chromatographically on PEI cellulose plates by TLC in 1.5M KH_2PO_4 , pH 3.4. The presented data were collected at an exponential phase of growth ($A_{600} = 0.3$) (b) Doubling time was determined spectrophotometrically with a plate reader. (c) Linear regression plot obtained by combining both parameters (generation time and alarmone accumulation), with R^2 values indicated. Results are obtained from at least three independent experiments and are expressed as the mean with SEM. Statistical significance was determined by unpaired Student's t-test.

2.5. Biofilm-Forming UPECs Are More Virulent to *Galleria Mellonella* Larvae

Finally, in order to determine if the biofilm-forming UPEC are more virulence than non-forming examples we employed a *G. mellonella* larvae model. As discussed in our previous work [38] this larvae serves as a surrogate model of infection. The model is cost

efficient, effective, reliable, and does not raise ethical concerns. Moreover, its use, has been described to study UPEC virulence [39,40]. The larvae were injected with a bacterial inoculum (10^4 to 10^7 cfu/larvae) to determine the mortality rate of insects exposed to each isolate. Five strains from each of the CRA+/- groups were tested. The survival of larvae was monitored for several days, and the results were collected in the form of Kaplan–Meier curves (Figure 5a). The *G. mellonella* larvae showed different survival profiles, depending on the strain used. However, in all cases, only the relatively high infectious dose 10^7 /larvae resulted in 100% mortality rate within 48 h. In addition, a quantified LD50 value between 24 and 72 h post-inoculation and the LD50 reduction level (LD50 72h/LD50 24 h) were calculated for each isolate (Figure 5b). The virulence of biofilm-forming isolates was higher in this model than that in the non-biofilm-forming samples.

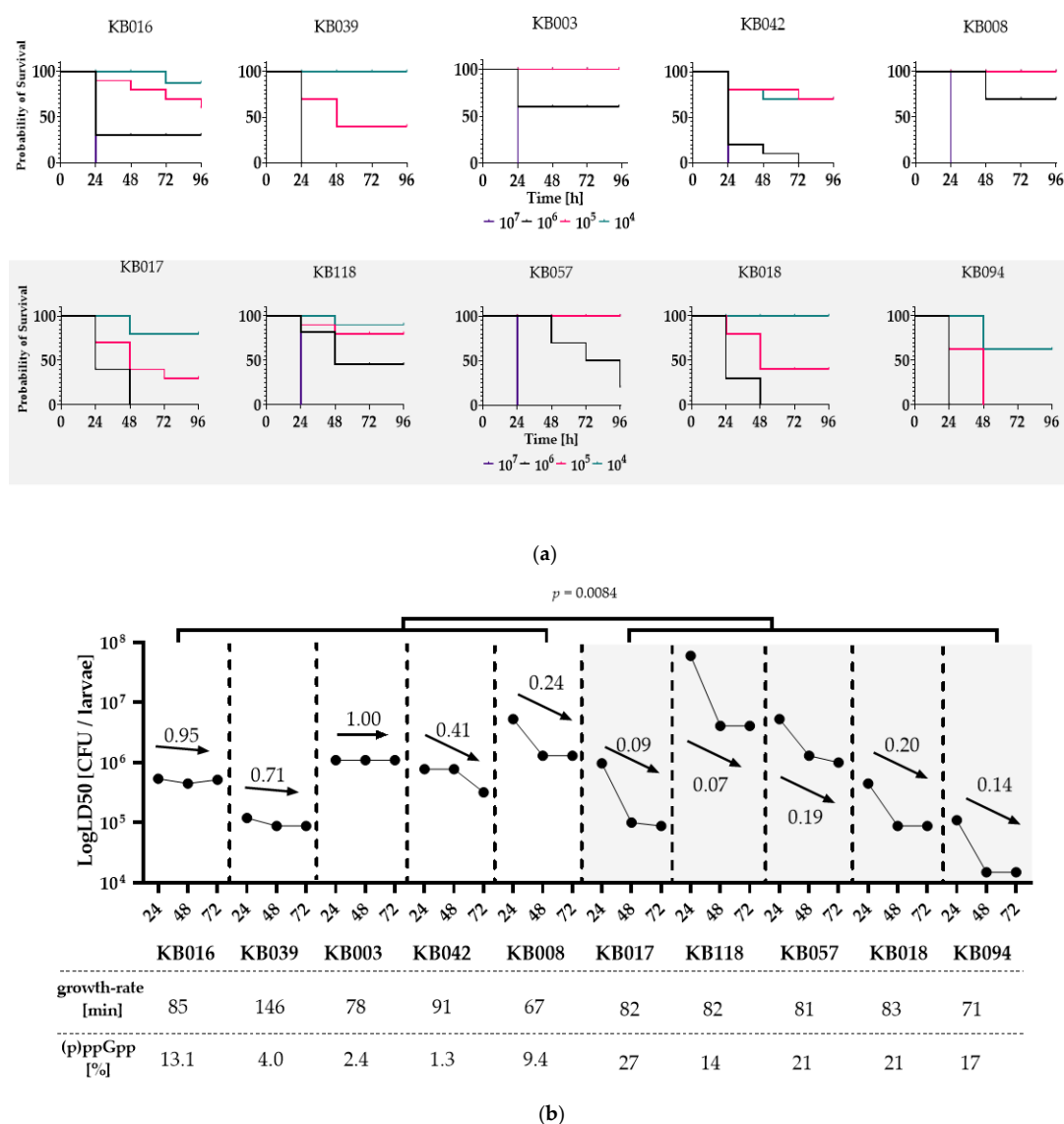


Figure 5. Evaluation of virulence of CRA+/- UPEC strains. Evaluation of virulence of biofilm-forming (gray part of panel) and non-biofilm-forming (colorless part of panel) UPEC strains in a surrogate infection model of *G. mellonella*. The 5 biofilm producers chosen were selected based on the greatest ability to form biofilm, and the 5 non-biofilm forming samples were randomly selected. (a) Kaplan–Meier survival curves of *G. mellonella* after injection of 10^4 – 10^7 cells/larva. (b) LD50 values calculated between 24 and 72 h. Percentage changes in LD50 between the 1st and 3rd days are indicated by arrows, with their value above them. The values of growth rate (min) and alarmone relative concentration (%) in particular strains are indicated under the plot.

3. Discussion

The goal of this work was to comprehensively characterize clinical isolates of the UPEC strains. Our studies show that these strains present characteristic features of their genus, but we also demonstrated a new characteristic phenotypic pattern for the studied collection, which should be further explored.

Among the 9 genes coding for the virulence factors tested, the most frequently detected were *fimH* (100%), *sitA* (92.5%), *aer* (75%), and *sfa* (70%). Such a high distribution of the *fimH* gene was also confirmed in studies conducted in Ethiopia, Romania, Mongolia, Iran, Mexico, and China [13,41–46], which may indicate a key role of this factor in the development of UTI. The percentage of other VFs (*Sfa*, *PapC*, *HlyA*, *CNF1*) that characterize the strains in the studied collection is on par with another study conducted in Poland [47], while in other countries, the share of these factors varies [13,48–52]. A high percentage of sequences encoding *SitA* and *Aer* characterized the UPEC studied, and this observation is in line with the results obtained in other studies [53–57]. The presence of such proteins indicates that, despite the environment in the urinary tract where iron access is limited, UPEC strains are able to survive and develop UTI. Additionally, the presence of the two genes in our study was significantly correlated with each other (Figure 2). Moreover, we observed a correlation between the genes of toxins related with a pathogenicity island, namely *hlyA* and *cnf*. The *cnf* gene was recently shown [58] to be dependent on *hlyA* presence, while *hlyA* occurrence is independent of *cnf*. The invasive protein *IbeA* and the serum survival enhancing factor *Iss* were the least frequent, with similar percentages described by Derachshandeh et al. [59]. The described virulence factors are crucial for triggering urinary tract infections; however, UPEC strains can differ significantly in terms of genetic background. This was confirmed by the fact that in the studied population of 120 strains, as many as 51 different virulotypes were obtained.

Pathotypes of virulent *E. coli* strains are often associated with a particular phylogroup. We determined that the predominant group in the collection was group B2 (71.7%), followed by groups B1 (10.8%) and D (7.5%). It is noteworthy that virulent extraintestinal strains mainly belong to groups B2 and D [60]. Similar findings were obtained in other studies, in which the B2 group was predominant [45,49,61–63]. Different phylogenetic groups expressed different average VF counts. Group B2 had the highest score of 6.01, groups A, C, and D were intermediate, while B1 had the lowest number of VF genes. A similar relationship for groups B2 and B1 was reported recently by Rezaatofghi et al. [64]. A positive correlation of VFs and those phylogroups was further observed in studies from the early 2000s [65,66].

The increasing prevalence of UTIs results in the overuse of broad-spectrum antibiotics, such as fluoroquinolones, cephalosporins, and aminoglycosides, which contributes to increased antibiotic resistance [10]. Resistant strains, including those not susceptible to more than one antibiotic, were also present in the population of isolates studied. The highest resistance was attributed to ampicillin (92.5%) and chemotherapy (norfloxacin, ciprofloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole; 58.5%). In their study, Oliveira et al. reported 59% of strains resistant to at least one of the antimicrobial agents tested, including the highest for ampicillin (51%) and trimethoprim-sulfamethoxazole (44%) [50]. Likewise, in an Australian study on antimicrobial use and bacterial resistance conducted in 2014–2015, the highest resistance rate for *E. coli* was also recorded for ampicillin (53.2%), followed by trimethoprim (31.3%). It is also worth mentioning that the percentage of resistant isolates was higher in 2015 when compared to 2014 [67]. A high rate of ampicillin-resistant strains was also obtained in other studies [47,52,68]. Currently, many strains resistant to multiple antibiotics remain susceptible to fosfomycin (FOS), even in geographic regions where its use is widespread. This antibiotic has shown efficacy, both in vitro and in vivo, in treating UTIs caused by ESBL-producing *Enterobacteriaceae* and vancomycin-resistant enterococci [69]. In this study we showed that none of the isolates, including the multidrug-resistant ones, were resistant to FOS (as well as to ceftibuten, meropenem, and ceftoxitin). Similar results with low rates of resistance or complete sensitivity to FOS have been obtained in other studies

at the Charlotte Maxeke Johannesburg Academic Hospital, among others [70–72]. These findings suggest that fosfomycin is still an effective treatment for urinary tract infections, but should be used with caution, and studies are needed to monitor the resistance status of strains isolated from patients with urinary tract infections.

It has been previously described that bacterial biofilm protects bacteria from antibiotics and is involved in many diseases [73]. Biofilms are communities of bacteria encased in a polymeric matrix that protects resident bacteria from both the antibiotics and the host immune effectors, among many other stressors and potential means of eradication. Our results confirm the correlation between biofilm-forming ability and antibiotic resistance phenotype; however this tested was conducted under non-biofilm-forming conditions. Although the percentage of resistant strains in the CRA+/- groups occurred at a similar level, biofilm-forming isolates were more likely to show resistance to more than one antibiotic. Bacteria residing within a biofilm are canonically highly resistant to antibiotic-mediated killing and are overall antimicrobial resistant, with resistance often exhibited toward multiple classes of antibiotics. Such a result was obtained in a study by Katongole et al. [74]. The authors also found no correlation of biofilm production with any of the virulence factors studied. Our study coincides with these observations, with the exception of the *ibeA* gene (this sequence was not studied in the Katongole et al. experiment). Furthermore, in another study conducted in Poland, no relationship between the rate of biofilm formation and the presence of adhesion factors or toxin genes was determined [47]. Only the aerobactin gene was significantly associated with strong biofilm production. However, it is worth mentioning that in this study, the assessment of biofilm formation capacity was investigated with a test other than CRA.

Biofilm is formed by the aggregation of bacterial cells, surrounded by a matrix composed of eDNA, proteins, and exopolysaccharides. These polysaccharides facilitate adhesion to the cell surface and protection from environmental stress. This is accompanied by a high concentration of 3', 5'-cyclic diguanosine monophosphate [c-di-GMP] [75]. Degradation of c-di-GMP occurs due to cyclic phosphodiesterase (PDE) activity of the YybT protein [76]. Interestingly, Rao et al. found that the bacterial regulator ppGpp is a potent inhibitor of the DHH/DHHA1 domain found in YybT, suggesting that YybT is under tight control during the stringent response [77]. In 2017, Jones et al. described that if c-di-GMP regulates the production of biofilm matrix polysaccharides, Congo red staining can be used as an indirect measurement of elevated c-di-GMP production in bacteria [78]. Hence, if elevated levels of (p)ppGpp contributes to increased levels of c-di-GMP, we concluded that strains with black colonies on CRA may produce increased levels of the alarmones. Our study confirmed the assumption that CRA+ strains have significantly higher basal levels of alarmones (Figure 4).

Moreover, in addition to the effect of (p)ppGpp on biofilm formation, alarmone production has also been linked to the expression of virulence factors or antibiotic tolerance. There are reports suggesting that UPEC strains with high intrinsic levels of (p)ppGpp exhibit higher levels of pathogenicity against the host [79,80]. To test whether reports of increased virulence of such strains would also be confirmed in our study, we screened the infectivity of UPEC differing in (p)ppGpp levels in a *G. mellonella* model. The results confirmed the initial assumption of increased virulence of CRA+ strains exhibiting higher intrinsic levels of (p)ppGpp (Figure 5). In laboratory *E. coli* strains, the cellular level of (p)ppGpp is closely related to their growth rate. The increase in the (p)ppGpp level is observed in response to stress factors, such as starvation, and results in a rapid accumulation of high alarmones concentrations (600–1000 pmol OD⁻¹) [81]. This leads to the inhibition of rRNA, DNA, and protein synthesis and in turn, yields slower growth rates. However, to date, there are still gaps in the knowledge regarding the role of the basal level of (p)ppGpp, which occurs in the cell under optimal growth conditions, and how it can affect cell physiology.

The high heterogeneity of the tested strains, demonstrated by genetic-phenotypic analysis, may arise from the differences in (p)ppGpp levels between clinical isolates. These

differences could be due to either the polymorphism of molecular targets of (p)ppGpp or proteins interacting with RelA and SpoT, including regulatory proteins or the polymorphism of RelA and SpoT proteins themselves. Stringent response exhibits pleiotropic effects, triggering vast metabolic adjustments in bacteria to adapt to a challenging environment. However, alarmones are also involved in VFs expression; therefore, we hypothesize that observe variations in (p)ppGpp levels among particular strains served as an adaptation mechanism, increasing competitiveness; however, this theory still awaits verification. Moreover, the observed common assumptions do not always correlate with the expected phenotype. As an example, enterohemorrhagic *E. coli* (EHEC) strains have a positive correlation between (p)ppGpp and the transcription of virulence factors encoded on the LEE pathogenicity island, while an inverse correlation is observed between high levels of (p)ppGpp and the Shiga toxin production [82]. Similarly, it is well known that high levels of (p)ppGpp result in low growth rates [30]. However, in this study, we observed an inverse correlation, as the biofilm-forming group of UPECs exhibited a significantly higher basal level of (p)ppGpp and, most remarkably, higher growth rates than the non biofilm-forming strains cultivated under the same non-limiting conditions, which is in stark contrast to the knowledge obtained to date. In a way, our observations are supported by a study on ECOR isolates (an evaluation of the regulatory relationship of (p)ppGpp and RpoS), which showed that regulatory pathways are not uniform within one species [83,84]. Experiments conducted on the K-12 strain suggest that (p)ppGpp should stimulate RpoS synthesis, but results described by Ferenci et al. indicate that RpoS levels are not equally stimulated by high (p)ppGpp in all ECOR isolates [84].

Furthermore, targets that are upregulated during induction of the stringent response may not be sensitive to basal ppGpp levels, which for some reason, are maintained at higher levels in the cell [85,86]. Imholz et al. described that modest increases in (p)ppGpp (<100 pmol OD⁻¹) do not appear to immediately inhibit biomass synthesis (except for stable RNA). High concentrations of pseudomonic acid rapidly increased ppGpp accumulation and canonically abruptly stopped growth. On the other hand, low concentrations of pseudomonic acid also triggered ppGpp synthesis (up to 60–100 pmol OD⁻¹) and an immediate, but smaller, decrease in rRNA synthesis. However, the exponential growth rate was not affected in the short term by a moderate increase in ppGpp concentrations [87]. The authors also determined that (p)ppGpp directly inhibits protein synthesis, controlling the rate of ribosome synthesis, albeit only at high concentrations of ppGpp. Potential direct inhibition of translation or ribosome-associated factors is probably not relevant at basal levels of (p)ppGpp [88].

Our results show that the role of (p)ppGpp in bacterial growth and virulence (tested in *G. mellonella* model) may be more complicated. The pleiotropic nature of the mechanism and its association with other regulatory pathways, as well as bacterial physiology and growth conditions, affect this correlation. Further research is needed to understand the relationship between basal (p)ppGpp levels and growth rate.

4. Materials and Methods

The strains were isolated and identified in the laboratory of Gdansk Regional Hospital in 2017 and 2021. All bacterial isolates data were anonymized. Of the 118 clinical *E. coli* strains, 93 were from women (78.8%) and 22 from men (18.6%). Patients' ages ranged from 1 month to 100 years. The reference uropathogenic strains UTI89 and CFT073 were used as controls, making the group size $n = 120$. Bacteria were grown in Lysogeny Broth (Lennox, Sigma-Aldrich, Darmstadt, Germany) at 37°C, with shaking. For growth rate assays, MOPS minimal medium with low phosphate (0.4 mM) was used. The optical density of cultures was monitored using an EnSpire instrument (Perkin Elmer Singapore Pte. Ltd., Singapore).

4.1. Virulence Gene Detection and Phylogenetic Group Determination

The UPEC collection was tested for the presence of virulence factors: FimH, Sfa, PapC, IbeA, HlyA, CNF1, Iss, SitA, the presence of Ag43 antigen, and phylogenetic group

membership, which were determined by PCR. All amplified products were visualized in 1% agarose in 0.5%TAE buffer (20 mM Tris pH 8.3, 0.5 mM EDTA, 10 mM acetic acid). RotiSafe dye (CarlRoth) was used to visualize the DNA. The details of primer (Sigma Aldrich) sequences and predicted sizes of amplified products are given in Table 4. UPEC isolates were assigned to separate groups (A, B1, B2, C, D, E, and F), according to Clermont's classification. The presence or absence of *arpA*/*chuA*/*yjaA*/TspE4.C2 genes was assessed using quadruplex PCR. Separate primers ArpAgpE and trpAgpC were used to determine allele-specific E and C phylogenies, respectively. PCR amplifications were performed in an Eppendorf TM Mastercycler proS thermocycler under the following conditions: initial denaturation at 94 °C for 4 min, 30 cycles of 5 s at 94 °C, and 20 s at 57 °C (E-group), or 59 °C (quadruplex and C-group), 72 °C for 1 min, and final extension at 72 °C for 5 min. Based on the genotypes obtained, the strains were grouped following the principles described by Clermont et al. in 2013 [17]. The presence of virulence factors encoding fimbriae P (*papC*), fimbriae type 1 (*fimH*), fimbriae S (*sfa*), cytotoxic necrotizing factor 1 (*cnf1*), hemolysin (*hlyA*), and aerobactin (*aer*) was determined by PCR, where reaction conditions included initial denaturation for 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 5 min at 72 °C, with a final elongation for 10 min at 72 °C. The reaction conditions for the *iss*, *sitA*, *ibeA*, and *agn43* primers were as described previously [53,89].

Table 4. Primers used in PCR reactions related to phylogenetic clustering and virulence gene detection.

PCR Reaction	ID	Target	Primer Sequence (5'-3')	Final Conc. (μM)	Product	Reference
Quadruplex	chuA.1b	<i>chuA</i>	5'-ATGGTACCGGACGAACCAAC-3'	20	288	[17]
	chuA.2		5'-TGCCGCCAGTACCAAAGACA-3'			
	yjaA.1b	<i>yjaA</i>	5'-CAAACGTGAAGTGTCAGGAG-3'		211	
	yjaA.2b		5'-AATGCGTTCCTCAACCTGTG-3'			
	TspE4C2.1b	TspE4.C2	5'-CACTATTCGTAAGGTCATCC-3'	40	152	
	TspE4C2.2b		5'-AGTTTATCGCTGCGGGTCGC-3'			
	AceK.f	<i>arpA</i>	5'-AACGCTATTCGCCAGCTTGC-3'		400	
	ArpA1.r		5'-TCTCCCCATACCGTACGCTA-3'			
Group E	ArpAgpE.f	<i>arpA</i>	5'-GATTCCATCTTGTCAAAATATGCC-3'	20	301	
	ArpAgpE.r		5'-GAAAAGAAAAAGAATTCCCAAGAG-3'			
Group C	trpAgpC.1	<i>trpA</i>	5'-AGTTTATGCCCAGTGCGAG-3'		219	
	trpAgpC.2		5'-TCTGCGCCGGTCACGCCC-3'			
Internal control	trpBA.f	<i>trpA</i>	5'-CGGCGATAAAGACATCTTCAC-3'	12	489	
	trpBA.r		5'-GCAACGCGGCTGGCGGAAG-3'			
Virulence genes	fimH F	<i>fimH</i>	5' TGCAGAACGGATAAGCCGTGG 3'	1	508	[90]
	fimH R		5' GCAGTCACCTGCCCTCCGGTA 3'			
	sfa F	<i>sfa</i>	5' CGGAGGAGTAATTACAAACCTGGCA 3'		407	[90]
	sfa R		5' CTCCGGAGAACTGGGTGCATCTTAC 3'			
	papC F	<i>papC</i>	5' GACGGCTGTACTGCAGGGTGTGGC 3'		328	[90]
	papC R		5' ATATCCTTTCTGCAGGGATGCAATA 3'			
	hlyA F	<i>hlyA</i>	5' AACAAAGGATAAGCACTGTCTGGCT 3'		1177	[90]
	hlyA R		5' ACCATATAAGCGGTCATTCCCGTCA 3'			
	aer F	<i>iucC</i>	5'-AAACCTGGCTTACCAACTGT-3'		269	[45]
	aer R		5'-ACCCGTCTGCAAATCATGGAT-3'			

Table 4. Cont.

PCR Reaction	ID	Target	Primer Sequence (5'-3')	Final Conc. (μM)	Product	Reference	
	cnf F	cnf	5'-TTATATAGTCGTCAGATGGA-3'		693	[45]	
	cnf R		5'-CACTAAGCTTTACAATATTGA-3'				
	iss F	iss	5' GTGGCGAAAAGTAAACAGC 3'		760	[53]	
	iss R		5' CGCCTCGGGTGGATAA 3'				
	sitA F	sitA	5' AGGGGGCACAAGTATTCTCG 3'		608	[53]	
	sitA R		5' TACCGGGCCGTTTCTGTGC 3'				
	ibeA F	ibeA	5' AGGCAGGTGTGCGCCGCTAC 3'		171	[53]	
	ibeA R		5' TGGTGCTCCGGCAAACCATGC 3'				
Multiplex PCR	CFT073a F	agn43aCFT073	5' AGGCAGGAGGAACTGCCAGT 3'	0.25	340	[89]	
	CFT073a R		5' TAAATGAGGGTGTCCTCGTGCC 3'				
	CFT073b F	agn43aCFT073	5' CAGCCGGATCTGCGGCACT 3'		440		
	CFT073b R		5' ACTCTGGTGTCTCTGGCTGTT 3'				

4.2. Evaluation of the Biofilm-Forming Phenotype

Biofilm formation of isolates was determined by culturing them on Congo red agar CRA plates, as described previously by Freeman et al. in 1989 [91] (Brain Heart Infusion Agar BHIA medium with 0.08% Congo red, supplemented with 3.6% sucrose). Briefly, bacteria were seeded on plates and then incubated at 37 °C for 24 h. The morphology of bacterial colonies was the basis of the categorization of the strain collection. Biofilm-producing strains formed black colonies, while non-biofilm-forming strains formed red colonies.

4.3. Susceptibility Assay

This evaluation was conducted by the disk-diffusion method using commercially available antibiotic disks. It was carried out in accordance with the European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines on Mueller–Hinton (MH) agar (Sigma Aldrich, Burlington, MA, USA) [92]. Briefly, a 0.5 McFarland unit (measured by a Densila-Meter II (ErbaLachema, Brno, Czech Republic)) suspension was prepared from overnight culture and spread on MH medium using a sterile swab. The plates were incubated at 37 °C for 24 h, and then zones of growth inhibition were measured. Susceptibility to 16 antibiotics (Biomaxima) was tested: amikacin (AK), nitrofurantoin (F), ampicillin (AM), ceftriaxone (CRO), norfloxacin (NOR), piperacillin (PRL), ciprofloxacin (CIP), gentamicin (CN), cefoxitin (FOX), cefuroxime (CXM), ceftibuten (CFB), meropenem (MEM), fosfomycin (FOS), azithromycin (AZM), trimethoprim/sulfamethoxazole (SXT), and tobramycin (TOB).

4.4. Evaluation of (p)ppGpp Accumulation

The intracellular alarmone levels were assessed according to the well-established TLC assay [93]. Briefly, bacterial colonies were suspended in MOPS (4-morpholinopropanesulfonic acid) minimal medium, with a low concentration of phosphate (0.4 mM), to a density of 0.5 by McFarland's standard, and then cultured for two generations with orthophosphoric acid [P^{33}] (0.5 μCi/mL). Samples were harvested over time and lysed with formic acid (13 M) and then frozen twice and centrifuged (5 min, 14000 RPM). Nucleotide extracts were separated by thin-layer chromatography using PEI cellulose plates (Sigma-Aldrich, Darmstadt, Germany) in 1.5 M potassium phosphate buffer (pH 3.4). Chromatograms were analyzed using a Phosphorimager (Typhoon 9200 GE Healthcare, Uppsala, Sweden). QuantityOne (BioRad, Hercules, CA, USA) software was used for densitometric analysis.

4.5. The Surrogate Model of Infection

Larvae of the greater moth *Galleria mellonella* (Lepidoptera: Pyralidae) were purchased from a livestock supplier (Exoticroom, Łódź, Poland) and were stored in the dark at

20 °C until use. In all experiments, larvae weighing about 250–300 mg, without signs of melanization and devoid of black spots in the epidermis, were used. The insects were infected with bacteria at a rate of 10^4 to 10^7 /larva using a Hamilton syringe with a blunt-ended needle for the injection in the last left proleg. Control group insects were injected with sterile PBS. The larvae were kept on petri dishes with Whatman blotting paper and incubated at 37 °C in the dark. Observations were made every 24 h for the next three days to detect changes in behavior and melanization. Death was determined by lack of movement and dark pigmentation of the larvae's epidermis.

4.6. Statistics

All experiments were performed in duplicate or triplicate. Statistical analyses were performed with Prism version 8.4.3 (GraphPad Software, San Diego, CA, USA) using Fisher's two-sided exact test, Pearson's test, and the Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

5. Conclusions

The results shown in this work present detailed the analysis of our UPEC strain collection. In addition to performing Clermont classification, we analyzed the ability of these strains to form biofilm and discovered a subset in the collection that effectively produced metabolically active biofilms. Most interestingly, isolates from this group exhibited increased intrinsic levels of the stringent response alarmones, (p)ppGpp, which was accompanied by higher growth rates. This finding is in contrast to the knowledge available to date regarding the role of (p)ppGpp in growth rate control. Our data also indicate that (p)ppGpp modulates biofilm formation and virulence in the *G. mellonella* model. Thus, the (p)ppGpp cellular pool is crucial for bacteria and links pathogenesis to their metabolic status.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms24043315/s1>.

Author Contributions: Conceptualization, D.N. and A.S.-P.; methodology, M.K. and D.N.; software, M.K. and D.N.; validation, M.K., P.S. and D.N.; formal analysis, M.K. and D.N.; investigation, M.K., P.S., K.B. and D.N.; writing—original draft preparation, M.K. and D.N.; writing—review and editing, M.K., D.N., A.S.-P. and K.P.; visualization, M.K. and D.N.; supervision, A.S.-P. and D.N.; funding acquisition, D.N. and A.S.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Gdansk, UGrants-start: 1220/29/2022 to D.N.; the National Science Center, Poland, within the SONATA grant (2018/31/D/NZ7/02258), to D.N.; and the National Science Center, Poland, within the OPUS grant (UMO-2016/21/B/NZ7/02077), to A.S.P.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank the staff of the Gdansk Regional Hospital for providing the *E. coli* strain collection. We are grateful to Natalia Barys for technical support.

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

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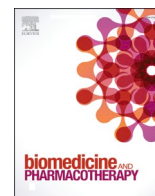
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Trans-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic *Escherichia coli*

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ARTICLE INFO

Keywords:

t-cinnamaldehyde
ExPEC
EPEC
Antimicrobial
Antivirulence
PpGpp
Biofilm

ABSTRACT

Antimicrobial resistance in *Escherichia coli* remains a critical threat in both human and veterinary medicine, demanding innovative strategies beyond traditional antibiotics. Here, we reveal that *trans*-cinnamaldehyde (*t*-CA), a natural phenylpropanoid, not only inhibits bacterial growth but also actively reshapes transcriptional programs through the RelA-mediated stringent response. Using RNA-seq, we demonstrate that sub-inhibitory *t*-CA (0.25 × MIC) triggers broad transcriptional remodelling, suppressing key virulence pathways such as iron acquisition, quorum sensing, and acid resistance - effects that were not pronounced in a *relA*-deficient background. Consistently, *t*-CA induced RelA-dependent accumulation of the stringent response alarmone, (p)ppGpp, linking stress signalling with repression of virulence determinants. Functionally, *t*-CA impaired extracellular matrix production and significantly reduced biofilm biomass and metabolic activity. In host-relevant assays, *t*-CA showed moderate cytotoxicity in mammalian cell lines and attenuated LPS-driven inflammatory cytokine output in Peripheral Blood Mononuclear Cells (PBMCs) model. Finally, post-infection administration of *t*-CA improved survival in the *Galleria mellonella* infection model. Together, these results identify stringent response signalling as a key axis underlying *t*-CA-driven virulence attenuation and support *t*-CA as a candidate antivirulence adjuvant for combating difficult-to-treat *E. coli* infections.

1. Introduction

Antimicrobial resistance (AMR) remains one of the most pressing challenges for both human and animal health. AMR undermines the effectiveness of first-line therapies and increases the risk of treatment failure across a broad range of bacterial infections [1]. Among the most problematic pathogens are extraintestinal pathogenic *Escherichia coli* (ExPEC), which drive a substantial proportion of urinary tract infections, bloodstream infections, and sepsis, and are increasingly associated with multidrug resistance and limited therapeutic options. In clinical settings, severe ExPEC infections can progress rapidly, particularly in infants, older adults, and immunocompromised patients, where bacteremia and systemic inflammation contribute to high morbidity and mortality.

Importantly, the AMR crisis is not confined to hospitals [2]. The selection and spread of resistance determinants are shaped by interconnected reservoirs spanning community settings, wastewater, wildlife, and food production systems. In agricultural environments,

frequent antibiotic exposure can select for MDR of multi-environmental bacteria such as *E. coli* and facilitate horizontal transfer of resistance genes within the gut microbiota and into the broader environment, accelerating dissemination across hosts and ecosystems. This is increasingly recognized as a One Health problem, in which clinically relevant resistance mechanisms can circulate between animal and human populations through shared microbial lineages, mobile genetic elements, and environmental interfaces. In parallel, pathogenic *E. coli* infections remain a major concern in livestock production; for example, colibacillosis in poultry and swine is a multifactorial disease that contributes to morbidity, mortality, and economic losses due to impaired growth and carcass condemnation [3,4]. These animal-associated infections are relevant not only for animal health but also because some *E. coli* populations in livestock may serve as reservoirs for zoonotic transmission and for resistance and virulence traits that can emerge in human clinical contexts [5–7]. As regulatory restrictions on the use of medically important antibiotics expand and stewardship intensifies [2,

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<https://doi.org/10.1016/j.bioph.2026.119306>

Received 28 January 2026; Received in revised form 21 March 2026; Accepted 27 March 2026

Available online 30 March 2026

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8], there is an urgent need for complementary interventions that reduce infection burden while minimizing selective pressure for stable resistance and preserving the effectiveness of existing antimicrobials [9].

New approaches in livestock production increasingly focus on reducing or replacing antibiotic use through alternative feed additives such as probiotics, prebiotics, organic acids, plant extracts, and vitamins, aiming to enhance animal health and performance while mitigating the risk of AMR [9]. Among these, phytochemicals have attracted growing interest due to their broad-spectrum bioactivity and relatively low risk of inducing resistance. One of the most extensively studied compounds is trans-cinnamaldehyde (*t*-CA), the major active constituent of cinnamon bark oil. *t*-CA exhibits both antimicrobial and antivirulence properties, including inhibition of bacterial quorum sensing, biofilm formation, and toxin production in various Gram-negative and Gram-positive pathogens. In addition to its direct effects on bacterial physiology, *t*-CA supplementation has been shown to positively influence host gut health. Animal studies indicate that dietary *t*-CA modulates the composition of intestinal microbiota, increases the abundance of beneficial commensals, and suppresses opportunistic pathogens [10, 11]. Furthermore, *t*-CA enhances the integrity of the intestinal epithelium by promoting tight junction protein expression and reducing markers of inflammation and oxidative stress [12,13]. These properties make *t*-CA a promising candidate for inclusion in functional feed formulations aimed at improving animal growth, resistance to infection, and overall welfare, particularly in antibiotic-free production systems.

Our recent work demonstrated that *t*-CA significantly downregulates the expression of key virulence genes, including *stx* encoding Shiga toxins, and disrupts central metabolic processes in enterohemorrhagic *E. coli* (EHEC) [14]. Importantly, the observed antivirulence effects appeared to be closely linked to bacterial stress responses mediated by the small nucleotide alarmones, namely guanosine tetra- and pentaphosphate alarmones ((p)ppGpp). These nucleotides are synthesized in *E. coli* by two enzymes, RelA, responding to amino acid starvation and SpoT, reacting to other stresses and limitation [15]. Stringent response regulatory system enables bacteria to reprogram global gene expression, balancing survival and virulence factor expression. The observed dysregulation of the stringent response in EHEC strains after *t*-CA treatment was linked to the induction of RelA synthase activity. Accumulation of (p)ppGpp molecules ultimately leads to attenuation of *stx* promoters transcriptional activity and inhibition of toxin production. Moreover, these effects were achieved without strong bactericidal activity, suggesting that *t*-CA may reduce virulence without inducing resistance. Investigating the molecular basis of *t*-CA action could provide broader insights into its utility as an alternative to conventional antibiotics in animal health, since the mechanisms controlling virulence and metabolic adaptation are often conserved across different *E. coli* pathotypes.

In this study, we assessed the susceptibility of 145 clinical and environmental *E. coli* isolates to *t*-CA and performed comparative transcriptomic profiling of wild-type *E. coli* and an isogenic Δ relA mutant exposed to sub-inhibitory *t*-CA to define pathways controlled by stringent response signalling. We complemented these data with functional readouts relevant to persistence and host interaction, including biofilm formation and extracellular matrix phenotypes, immunological profiling in peripheral blood mononuclear cells (PBMCs), and post-infection efficacy testing in the *Galleria mellonella* model. Together, this integrated framework connects *t*-CA-induced transcriptional reprogramming with functional outcomes that shape *E. coli* fitness and pathogenic phenotype, including biofilm formation and host-associated responses. By showing how a dietary phytochemical can steer bacterial stress regulation toward attenuation of virulence-linked traits, our findings support phytochemical-based antivirulence strategies as practical adjuncts to conventional therapy. Such approaches may help decreasing the required antibiotic exposure, preserve treatment efficacy, and ultimately reduce the selective pressure that drives resistance emergence and spread.

2. Methods

2.1. Growth conditions and susceptibility evaluation

The antibacterial activity of trans-cinnamaldehyde (*t*-CA, 97%, Sigma Aldrich, C80687) was evaluated by determining the minimum inhibitory concentration (MIC) according to the CLSI M07-A9 guidelines [16]. Bacterial suspensions were prepared from fresh overnight agar cultures and adjusted to a turbidity equivalent to 0.5 McFarland standard. MIC testing was performed using the standard broth microdilution method in Mueller–Hinton (MH) broth on 96-well plates, with *t*-CA concentrations ranging from 15 to 0.117 mM. After overnight incubation at 37 °C, aliquots from each well were plated onto Mueller–Hinton agar (MHA) to determine the minimum bactericidal concentration (MBC). Bacterial strains (n = 145) used in the study are listed in Table S1. Additionally, the antimicrobial activity of *t*-CA was assessed using a classical agar disk diffusion assay for *E. coli* MG1655, MG1655 Δ relA, and MG1655 Δ relA Δ spoT strains. Bacterial lawns were prepared on MHA plates using suspensions standardized to 0.5 McFarland, followed by application of sterile paper disks (6 mm) loaded with *t*-CA. Plates were incubated at 37 °C for 24 h, and the diameters (mm) of inhibition zones were measured. Bacterial growth curves were monitored spectrophotometrically at 600 nm (OD₆₀₀) using a 96-well microplate reader (EnSpire, Perkin Elmer, Singapore). Fresh overnight cultures of *E. coli* MG1655, MG1655 Δ relA, and MG1655 Δ relA Δ spoT were diluted in LB medium supplemented with *t*-CA at concentrations of 3.75 mM, 1.875 mM, and 0.9375 mM. Cultures were incubated at 37 °C with continuous shaking, and growth was recorded over time.

2.2. Intracellular (p)ppGpp assessment

Intracellular levels of (p)ppGpp were determined following the thin-layer chromatography (TLC) method [17]. Briefly, bacterial strains were initially grown on LA plates and subsequently suspended in MOPS minimal medium containing reduced phosphate concentration (0.4 mM), adjusted to a turbidity of 0.5 McFarland. Cells were labelled with [³P]-orthophosphate (1 μ Ci/mL; Hartmann Analytics GmbH) and incubated for two generations. At time point 0, cultures were treated with *t*-CA at final concentrations of 3.75 mM, 1.875 mM, or 0.9375 mM. Control cultures were treated with serine hydroxamate (SHX) to induce (p)ppGpp alarmone accumulation. Untreated samples were used as negative control. Samples were collected at 15, 30, and 60 min post-treatment. Untreated samples served as controls. Cells were lysed using 13 N formic acid, followed by two freeze–thaw cycles, and centrifuged at 14,000 $\times$ g for 5 min. Supernatants containing nucleotides were separated by TLC on polyethyleneimine (PEI)-cellulose plates using 1.5 M potassium phosphate buffer (pH 3.4) as the mobile phase. Radiolabelled spots were visualized using a Typhoon 9200 Phosphor-imager (GE Healthcare), and densitometric analysis was performed using QuantityOne software (Bio-Rad).

2.3. RNA isolation and RNA-seq based transcriptomic analysis

The experiment was conducted as previously described [14], with modifications. Overnight cultures of *E. coli* MG1655 and MG1655 Δ relA were diluted 1:100 in LB medium and incubated at 37 °C until reaching an OD₆₀₀ of 0.1. At this point, *t*-CA was added to a final concentration of 0.9375 mM. Samples were collected at 30 and 60 min post-treatment and stabilized by the addition of 5% AquaPhenol in 96% ethanol. Cells were snap-frozen in liquid nitrogen and stored at –20 °C. Total RNA was extracted after thawing and centrifugation by resuspending the cell pellet in TE buffer containing lysozyme, followed by purification using the RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's protocol. RNA quality and integrity were assessed using the Agilent 2100 Bioanalyzer. RNA sequencing was performed using the NovaSeq 6000 platform (Macrogen Europe).

2.4. RT-qPCR gene expression analysis

Total RNA isolated as described above was used for quantitative reverse-transcription PCR (RT-qPCR) according to the protocol of Karczewska et al. Karczewska et al., [14]. First-strand cDNA synthesis was performed with the SuperScript™ III First-Strand Synthesis Super-Mix for RT-qPCR (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. Quantitative PCR assays were carried out for *E. coli* MG1655 and its *relA* mutant using the primer pairs and cycling conditions as presented in Table 2. Each reaction was run in triplicate technical replicates. Relative transcript levels were calculated with the $\Delta\Delta C_t$ method.

2.5. Gene ontology evaluation

RNA-seq data were analyzed according to the previously described workflow [14], including quality control with FastQC, read mapping to the *E. coli* MG1655 reference genome (Ensembl Bacteria 57) using the Galaxy platform (usegalaxy.org), and read counting with FeatureCounts. Differential gene expression analysis was performed using the DESeq function in RStudio (version 4.3.1). Genes with a minimum fourfold change in expression ($|\log_2 FC| \geq 2$) and a statistical significance threshold of $p < 0.05$ were considered differentially expressed. Comparisons were made between samples collected at 30 and 60 min post *t*-CA treatment relative to time point zero. Enrichment analysis of differentially expressed genes (DEGs) was carried out using the DAVID platform (Database for Annotation, Visualization and Integrated Discovery), focusing on Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [18]. Protein-protein interaction analysis was performed using STRING v12.0 (Search Tool for the Retrieval of Interacting Genes/Proteins) [19].

For transcriptome-wide visualization, ridgeline (joyplot) plots of $\log_2 FC$ values across all genes were generated using bioinformatics.com.cn. Additional figures were prepared using GraphPad Prism software.

2.6. Cytotoxicity assessment

Human dermal fibroblasts (HDFa) were obtained from Cascade Biologics, whereas human mammary epithelial HB2 cells were provided by Dr. R. Sadej from the Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Poland. HDFa and HB2 cell lines were cultured as adherent monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and a standard penicillin-streptomycin antibiotic solution. In the case of HB2 cells, the culture medium was further supplemented with hydrocortisone (5 µg/mL) and bovine insulin (10 µg/mL). All cultures were maintained at 37 °C in a humidified incubator under a 5% CO₂ atmosphere.

Cell viability was assessed using the MTT assay according to standard procedure [20]. Cells were seeded in 96-well plates at a density of 4×10^3 cells per well and incubated overnight to allow for attachment.

Table 1
Inhibitory concentrations of *trans*-cinnamaldehyde (*t*-CA) MIC and MIC₉₀ against *E. coli* strains.

Strain	Pathotype	Inhibitory concentration (MIC)*	MIC ₉₀ **
MG1655	-	3.75	
MG1655Δ <i>relA</i>	-	1.86	
MG1655Δ <i>relA</i> Δ <i>spoT</i>	-	1.86	
CFT073	UPEC	3.75	
UTI89	UPEC	3.75	
86-24	EPEC	1.86	
EDL933	EPEC	1.86	
<i>E. coli</i> (n = 145)			3.75

* Inhibitory concentrations (MIC) were presented in mM
** Minimal inhibitory concentration active against 90 % of strains tested

Table 2
Primers used in RT-qPCR.

Name	Gene target	Sequence 5'-3'
mdh_F	<i>mdh</i>	AGCGCGTAAACCGGTTATGG
mdh_R	Malate dehydrogenase	CGCTTTCGGGCGAGTTTTCG
sdhB_F	<i>sdhB</i>	CGAGACTGACAGCCGCTC
sdhB_R	Succinate dehydrogenase	GGTTCAGCCCCCTTCGGACAT
adhE_F	<i>adhE</i>	ACGTGTTTCACAGTGACGCGA
adhE_R	Aldehyde-alcohol dehydrogenase E	TTTGCCAGACGGTGCGGAAT
rplW_F	<i>rplW</i>	ACGTACCGACACGCGTATCG
rplW_R	Large ribosomal protein L23	TTACTCAGCGCCGCAACG
fliA_F	<i>fliA</i>	GTCACGAAGCATTGCGCCTG
fliA_R	Flagellar specific sigma factor σ28	AATGCCGTTCTGTAGGGCG
fliC_F	<i>fliC</i>	AATGGCGTCGATCGCGTGAT
fliC_R	Flagellar transcriptional regulator C	CAATGTCCAGGCACCGTT

*qPCR amplification cycles: (i) 95°C - 10 s; (ii) 60°C - 10 s; (iii) 72°C - 30 s. This programme was repeated 30 times, and preceded by an initial denaturation step at 95 °C for 5 min.

The following day, the culture medium was replaced with fresh medium containing various concentrations of *t*-CA, and cells were incubated for 24 h. Prior to the end of the treatment period, 25 µL of MTT solution (4 mg/mL) was added to each well. After a 3-hour incubation, the supernatant was removed, and the resulting formazan crystals were solubilized in 100 µL of DMSO. Absorbance was then measured at 570 nm with a reference wavelength of 660 nm using a BioTek microplate reader (Agilent Instruments). All data represent the mean of at least three independent experiments, each performed in triplicate.

2.7. Isolation, culture and assessment of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy blood donors (Regional Centre for Blood Donation and Blood Treatment in Gdańsk, Poland; consent number: 04/2025). Isolation was performed as previously described [21] by density gradient centrifugation using Ficoll (1.077 g/L; Sigma-Aldrich, Saint Louis, MO, USA). Following isolation, cells were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Biowest, Nuaille, France), supplemented with 10% fetal bovine serum (FBS; Biowest), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, Saint Louis, MO, USA), and seeded at a density of 2×10^6 cells/mL. PBMCs were allowed to equilibrate for 24 h prior to compound exposure.

After the initial 24 h incubation period, PBMCs were treated for 24 h with increasing concentrations of *t*-CA. Following exposure, cells were collected and stained with 0.4% Trypan Blue solution (Thermo Fisher Scientific, Waltham, MA, USA). Viable and non-viable cells were quantified using the LUNA™ II automated cell counter (Logos Biosystems, Anyang, South Korea).

Cytokine production was assessed in PBMCs following stimulation with LPS (*Escherichia coli* O111:B4, Sigma-Aldrich, Saint Louis, MO, USA) and subsequent post-treatment with *t*-CA. PBMCs were seeded at a density of 2×10^6 cells/mL and obtained from four independent donors, with each condition analyzed in three technical replicates. Cells were stimulated with LPS (100 ng/mL) and then exposed to the tested compound. IL-1β, IL-6 and TNF-α were quantified after 2 h LPS + 4 h compound post-treatment, and IL-10 after 4 h LPS + 20 h compound post-treatment. Cytokine levels in supernatants are presented as mean ± SD. Quantification of cytokine levels was performed using Human DuoSet® ELISA Development Systems (R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer's instructions. Technical replicates were averaged per donor. Independent donors (n = 4) were treated as matched biological replicates and analyzed using the Friedman test with planned post hoc comparisons versus LPS (Dunn's test; α = 0.05).

2.8. Biofilm formation

Biofilm macrocolony formation was initiated by plating 5 mL of bacterial suspension (colonies suspended in LB medium without salt, 5 g/l yeast extract and 10 g/l tryptone) on YESCA agar (10 g/l casein acids, 1 g/l yeast extract and 20 g/l agar). To visualize curli and cellulose, 10 µg/mL Calcofluor White (CF) or 50 µg/mL Congo Red (CR) were added to the media (brilliant blue 1 µg/mL). Plates with bacterial cultures were incubated at 26 or 37 °C for 24 h. After this time, morphology, colony color and fluorescence visualized under UV light were evaluated and photographed. For the glass tube test, overnight bacterial cultures were diluted 1:100 in LB without salt in glass tubes. The tubes were incubated without shaking at 26 °C. Observations were carried out after 72 h.

Quantitative analysis of *t*-CA impact on biofilm mass and viability was carried out using 96-well microplates cultures. Bacterial biofilms treated or untreated (controls) with indicated agents were then assessed by additional staining procedures using PBS solutions of crystal violet (1.0%) or formazan salt (0.1%, MTT) to evaluate the total mass or metabolic activity, respectively. Tetracycline (TET) and fosfomycin (FOS) were applied as positive controls, and used at 1 × and 0.5 × MIC (as determined under the same assay conditions). Results were developed spectrophotometrically (A_{570} or A_{575}), data were normalized

2.9. In vivo activity evaluation *Galleria mellonella*

Assessment of bacterial virulence using insect model was performed as described in [14]. Briefly, healthy larvae (weight of 250–300 mg, without signs of melanization and devoid of black spots in the epidermis) of the greater wax moth *G. mellonella* purchased from Exotricom, (Łódź, Poland) were stored in the dark at 20 °C prior to use. For the assessment of treatment efficacy, the insects were infected with bacteria at a concentration of 1×10^6 cfu per larva using a Hamilton syringe with a blunt-ended needle into the last left proleg. After one hour of infection, *t*-CA (6.25–50 mg per kg of body weight) were administered (10 µl) to the insects in the last right proleg. Control larvae were injected with PBS. The larvae were placed on Petri dishes with Whatman filter paper and incubated in darkness at 37 °C. Observations were carried every 24 h for the following four days, monitoring changes in behavior and melanization. Death was determined by the lack of movement and dark pigmentation of the larvae's epidermis. The procedures were repeated three times with $n = 10$ per group. On the basis of one representative experiment, the Kaplan–Meier survival curves were prepared.

2.10. Data availability

Transcriptomic data from this work can be found at Gene Expression Omnibus under accession number GSE252441 and GSE301628.

2.11. Statistical analysis

All experiments were performed in at least three independent biological replicates, unless stated otherwise. Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM), as indicated in figure legends. Comparisons between two groups were evaluated using Student's *t*-test (unpaired, two-tailed) or one-way ANOVA (≥ 3 groups). Statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) and RStudio (version 4.3.1) for transcriptomic datasets. For RNA-seq data, statistical significance for differential gene expression was determined using the DESeq2 algorithm with Benjamini–Hochberg correction for false discovery rate (FDR). A *p*-value of less than 0.05 was considered statistically significant in all analyses

3. Results

3.1. Antibacterial activity of *t*-CA against *E. coli* and pathogenic isolates

To explore the antimicrobial potential of *t*-CA against *E. coli*, we assessed its inhibitory and bactericidal activity in both laboratory and clinically relevant backgrounds. MIC and MBC values were determined for the reference strain *E. coli* MG1655 as well as its isogenic $\Delta relA$ and $\Delta relA \Delta spoT$ mutants, which lack key components of the stringent response. These strains allowed us to examine whether disruption of global stress regulation modulates susceptibility to *t*-CA (Table 1, S1).

In parallel, we evaluated a panel of pathogenic isolates representing two clinically and veterinary significant pathotypes: uropathogenic (ExPEC) and enteropathogenic (EPEC) *E. coli* (Table 1, S1). These strains are commonly implicated in extraintestinal infections across host species, including companion and farm animals. Remarkably, all strains (regardless of origin or genotype) exhibited a narrow MIC distribution, with an MIC₅₀ of 3.75 mM. This uniform susceptibility pattern suggests that *t*-CA maintains its antimicrobial activity across diverse *E. coli* backgrounds, including strains with distinct virulence repertoires and stress response capacities. These results suggest that *t*-CA exhibits consistent antibacterial activity against a range of *E. coli* strains, including both laboratory and pathogenic isolates, and is not strongly affected by strain-specific virulence or resistance factors.

3.2. Analysis of stringent response in *t*-CA susceptibility

The antibacterial effects of *t*-CA on *E. coli* MG1655 and its $\Delta relA$ and $\Delta relA \Delta spoT$ mutants were evaluated using multiple experimental approaches, as summarized in Fig. 1. In the agar diffusion assay with *t*-CA-impregnated paper disks, clear zones of inhibition were observed around all test disks (Fig. 1a). The diameter of these zones varied between strains, with the largest inhibition zone detected for the $\Delta relA \Delta spoT$ double mutant (36 ± 1.0 mm), $\Delta relA$ (28 ± 0.8 mm) and WT (27 ± 1.1 mm). To assess bacterial growth in liquid culture, optical density was monitored in the presence of increasing concentrations of *t*-CA (Fig. 1b). All three strains exhibited dose-dependent growth inhibition, with the $\Delta relA \Delta spoT$ mutant displaying the highest sensitivity. However, it is well established that deletion of both *relA* and *spoT* results in a complete loss of (p)ppGpp synthesis, impairing the cell's ability to adapt to environmental stress and leading to generally reduced growth dynamics [22]. This phenotype was reflected in the growth curves, where the wild-type strain demonstrated more robust proliferation than the stringent response-deficient mutant. To quantitatively compare the inhibitory effect of *t*-CA, the area under the growth curve (AUC) was calculated and converted into percentage inhibition relative to the untreated control (Fig. 1c). In *E. coli* MG1655, *t*-CA at MIC and sub-MICs (3.75 and 1.875, 0.9375 mM) reduced growth by 99.99%, 39.56%, and 12.43%, respectively. In the $\Delta relA$ mutant, the corresponding inhibition values were 99.97%, 57.77%, and 7.88%, while in the $\Delta relA \Delta spoT$ strain observed inhibition reached 99.9%, 99.5%, and 50.5%. These results confirm the dose-specific sensitivity of the strains to *t*-CA. To further investigate the underlying mechanism, intracellular levels of (p)ppGpp were measured following *t*-CA exposure (Fig. 1d). In the wild-type strain, *t*-CA induced a concentration-dependent increase in (p)ppGpp levels, indicating activation of the stringent response. In contrast, (p)ppGpp remained undetectable in both $\Delta relA$ and $\Delta relA \Delta spoT$ strains, regardless of *t*-CA concentration. This confirms that *t*-CA-induced activation of the stringent response operates via RelA-dependent (p)ppGpp synthesis.

Taken together, these results suggest that *t*-CA interferes with bacterial metabolism, at least in part by triggering the stringent response through RelA. However, significant growth inhibition was still observed in all strains, including those lacking both RelA and SpoT, indicating that *t*-CA also exerts RelA-independent antibacterial effects.

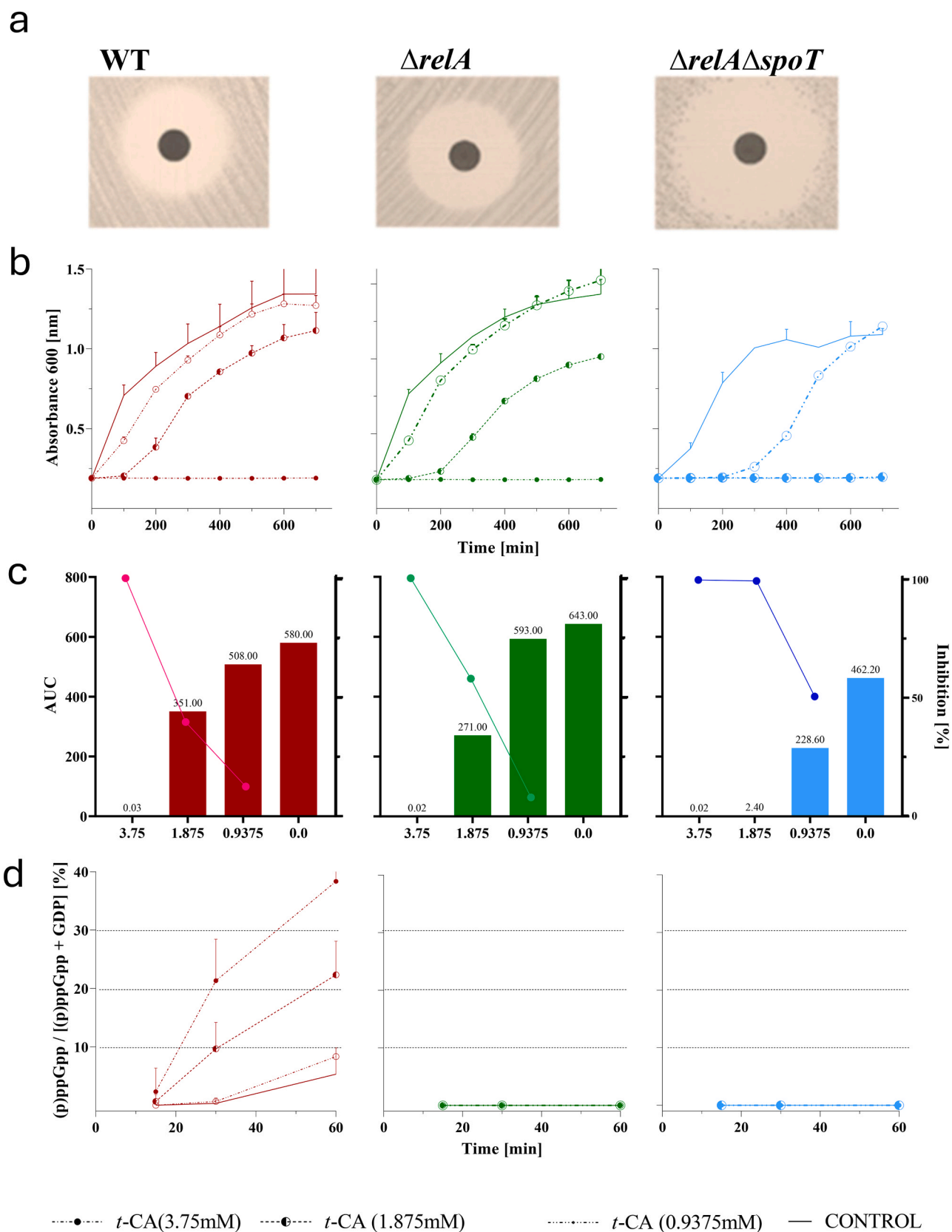


Fig. 1. Antimicrobial activity *t*-CA against *E. coli* WT, *relA* and *relA* $\Delta spoT$ deficient strains. (a) Representative images of growth inhibition zones around *t*-CA-impregnated discs. (b) Dose-dependent bacterial growth curves showing optical density (OD_{600}) over time under *t*-CA treatment at 0.9375 mM, 1.875 mM, and 3.75 mM compared to untreated control. (c) Quantitative analysis of growth inhibition expressed as area under the curve (AUC) (left axis) and corresponding percentage of inhibition relative to control (right axis). (d) Intracellular levels of (p)ppGpp measured over time after *t*-CA exposure, highlighting *RelA*-dependent induction of the stringent response. All data represent the mean of at least three independent experiments, each performed in triplicate.

3.3. RNA-sequencing and quality control

Next, to investigate the global transcriptomic changes induced by *t*-CA, RNA-sequencing was performed on WT and $\Delta relA$ strains of *E. coli* exposed to $0.25 \times \text{MIC } t\text{-CA}$ for 30 and 60 min. The concentration of *t*-CA was selected to minimize confounding effects of growth arrest while preserving regulatory responses. RNA sequencing generated over 40 million raw reads per sample (ranging from 40,152,336–59,741,406) in both *t*-CA-treated and control groups (Dataset2). The Q30 %age for each library ranged from 93.71% to 94.69%, indicating high sequencing quality. Quality control analysis confirmed strong consistency among biological replicates. Density plots of z-transformed expression values (Figure S1a) revealed that transcript distribution profiles shifted over time in both strains, with WT showing more compact and time-dependent transcriptional modulation. In contrast, the $\Delta relA$ mutant exhibited broader, less structured expression profiles, suggesting impaired regulation in the absence of stringent response. Principal component analysis further confirmed these observations. In the WT background, control and *t*-CA-treated samples formed distinct clusters along PC1 (63.34%), with a clear separation between 30 and 60 min treatments (Figure S1b). In the $\Delta relA$ strain, assessment of PC1 and PC2 accounted for a 49.8% and 24.1% of variance respectively, with samples also forming discrete clusters by treatment time (Figure S1c), although with increased replicate dispersion. These results suggest that *t*-CA induces a global and time-resolved transcriptomic shift in *E. coli*, and that the presence of RelA is required for a more coordinated gene expression response under phytochemical stress.

3.4. Transcriptional profile analysis

To identify differentially expressed genes (DEGs) following *t*-CA treatment, pairwise comparisons were performed for each strain and time point using DESeq2 (Fig. 3). Volcano plots (Fig. 2a) revealed a substantial number of genes significantly affected by *t*-CA exposure ($\text{padj} < 0.05$, $|\log_2 \text{FC}| > 2$) in both WT and $\Delta relA$ strains. In WT cells, the number of upregulated and downregulated genes increased between 30 and 60 min (459 and 569 DEGs, respectively), indicating a time-

dependent response. Notably, the $\Delta relA$ mutant showed an even broader transcriptional response, with a higher number of both induced and repressed transcripts (992 and 1016 DEGs, in 30 and 60 min respectively) at each time point compared to the WT. This suggests that RelA may act to buffer or refine the transcriptional response to stress, preventing excessive or unspecific gene activation. In the absence of RelA, the transcriptomic changes appear more extensive and potentially less coordinated.

As presented on Venn diagram (Fig. 2b) while 154 genes were commonly regulated across all experimental groups, a large number of DEGs were unique to specific conditions, particularly in the $\Delta relA$ background (e.g., 489 genes unique to $\Delta relA$ at 30 min). In contrast, WT strains shared more DEGs across time points, consistent with a more progressive and regulated shift in gene expression. These results indicate that the stringent response significantly influences the amplitude and specificity of the transcriptional adaptation to *t*-CA.

3.5. GO functional enrichment analysis

Functional analysis revealed that the transcriptional response to *t*-CA in the $\Delta relA$ mutant was dominated by genes associated with core cellular processes such as ribosome assembly, translation machinery, and protein translocation (Fig. 3a). This strong representation of translation-related functions points to a loss of typical growth regulation, where transcription of ribosomal components is normally repressed under stress. In the absence of (p)ppGpp, cells appear to remain in a “growth-ready” state, even in the face of phytochemical challenge, which is in the agreement of fundamental observations of decreased survival of (p)ppGpp-devoid bacteria under stress (PMID: 2005134). Despite comparable growth inhibition observed in both wild-type and $\Delta relA$ strains exposed to subinhibitory concentrations of *t*-CA, their transcriptional responses revealed fundamentally different strategies. In WT cells, *t*-CA triggered a downregulation of virulence-associated pathways, including quorum sensing, motility systems, and iron acquisition (enterobactin biosynthesis). These changes suggest a strategic shift from aggressive and energy-consuming colonization toward stress endurance. In contrast, the $\Delta relA$ mutant, though equally sensitive to *t*-

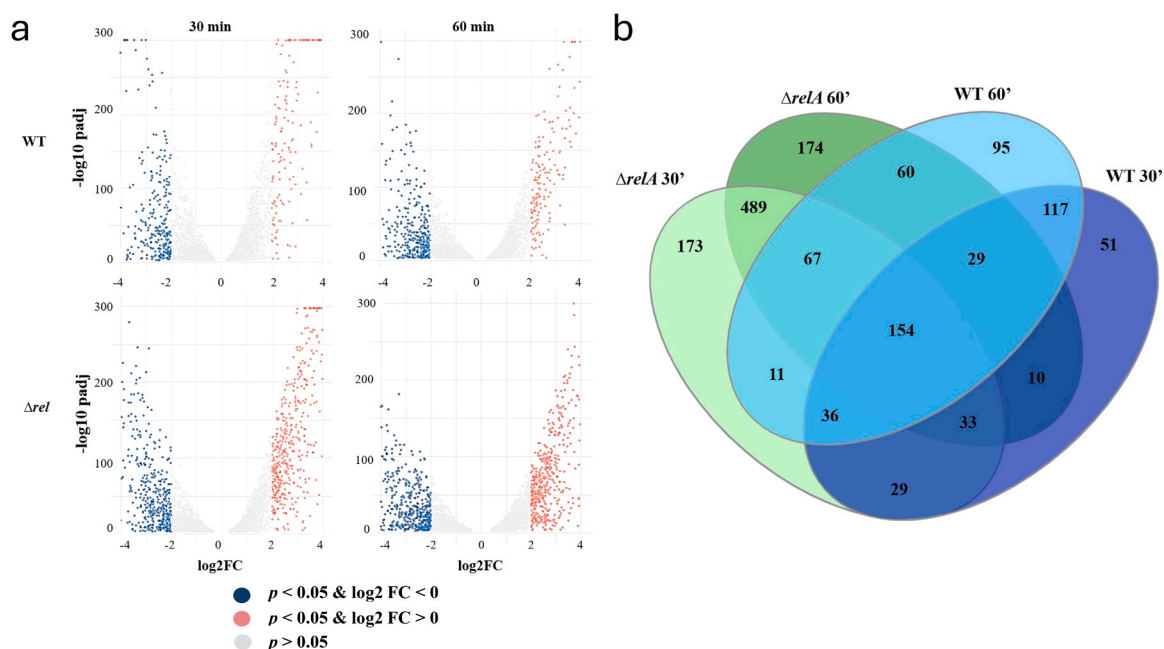


Fig. 2. Differential gene expression in *E. coli* WT and $\Delta relA$ strains in response to *t*-CA. Volcano plots showing $\log_2$ fold change versus $-\log_{10}$ adjusted p-values for WT and $\Delta relA$ strains at 30 and 60 min post *t*-CA exposure (a). Genes significantly upregulated (red) or downregulated (blue) were defined by $\text{padj} < 0.05$ and $|\log_2 \text{FC}| > 2$. Grey points indicate non-significant changes. The Venn diagram (b) displays the overlap of differentially expressed genes (DEGs) between all four conditions. Numbers represent genes commonly or uniquely regulated under each treatment.

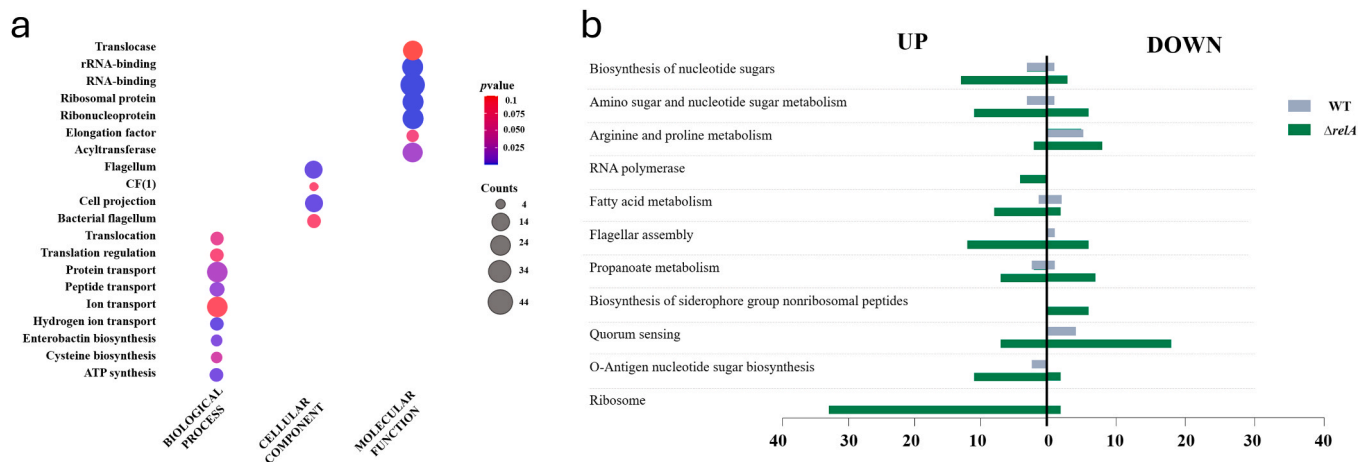


Fig. 3. Functional enrichment analysis of differentially expressed genes (DEGs) in *E. coli* WT and $\Delta relA$ strains in response to *t*-CA treatment. (a) Gene ontology (GO) enrichment for upregulated DEGs in the $\Delta relA$ strain after 60 min of *t*-CA exposure. Terms are grouped by GO category: Biological Process, Cellular Component, and Molecular Function. Circle size represents the number of enriched genes; colour indicates statistical significance (adjusted p-value). (b) KEGG pathway enrichment analysis comparing up- and downregulated pathways in WT (grey) and $\Delta relA$ (green) strains. Bars represent the number of genes associated with each enriched pathway. Notable differences were observed in siderophore biosynthesis, quorum sensing, and ribosomal pathways.

CA at the level of proliferation, failed to suppress these virulence programs. Functional enrichment showed an increase in expression of ribosomal genes, flagellar assembly components, and biosynthetic pathways - traits typically curtailed during environmental stress. This negatively affecting cell survival scenario results from the absence of (p)ppGpp signalling (so-called relaxed response), which in WT enables transcriptional reallocation from growth to defence.

Pathway-level enrichment (Fig. 3b) confirmed upregulation of ribosomal and metabolic modules, consistent with the lack of (p)ppGpp-mediated repression in the absence of RelA. The data point to a scenario where *E. coli* lacking RelA remains vulnerable to *t*-CA-induced stress but lacks the transcriptional capacity to shift from growth to defence mode. As a result, while bacterial proliferation is affected, among other energy-utilizing processes, effective virulence program can be active, potentially maintaining pathogenic potential. These findings position RelA not only as a modulator of global transcriptional dynamics, but as a necessary gatekeeper for the full expression of *t*-CA's antivirulence potential.

3.6. *t*-CA mediated transcriptomic rearrangement is controlled by RelA activity

To gain deeper insight into RelA-dependent transcriptional remodelling, we analysed selected functional gene sets associated with translation, central metabolism, pH regulation, and iron homeostasis as presented on Figures S2 and 4. In the $\Delta relA$ background, we observed a pronounced upregulation of ribosomal protein genes (*rps*, *rpl*, *rpm* operons), consistent with the loss of (p)ppGpp-mediated repression of translation under stress (Figure S2ab). This supports the notion that RelA is essential for stress-induced growth arrest at the transcriptional level in concert with the canonical mode of action of the stringent response. Additionally, we detected significant upregulation of the *rpoABC* operon in the $\Delta relA$ strain, with fold changes of 3.3, 3.9, and 3.9, respectively, while expression of these genes remained stable in the wild-type strain under the same treatment conditions (Dataset 1). This upregulation was accompanied by increased expression of several transcriptional regulators, including *tufA* (elongation factor EF-Tu), *gyrB* (DNA gyrase subunit), and *greA* (transcription elongation factor), all of which were downregulated or unchanged in the wild type (Dataset 1).

Meanwhile, several genes involved in central metabolism, particularly those linked to the acetate and TCA cycle pathways (e.g. *aceE*, *aceF*, *poxB* and *sdhA*), were downregulated, indicating that $\Delta relA$ cells may

shift away from efficient energy production under phytochemical stress (Fig. 4ab). This metabolic imbalance may reflect the lack of global resource reallocation normally imposed by the stringent response.

Striking differences were also observed in the regulation of pH homeostasis genes (Fig. 4ab). In WT, genes of the glutamate-dependent acid resistance system (*gadW*, *gadX*, *gadC*) and lysine decarboxylase pathway (*cadA*) were moderately induced, whereas in $\Delta relA$, a distinct pattern emerged with strong induction of alternative systems such as *yfiR* and *phoU*. This suggests that *relA*-deficient cells activate compensatory pH-stabilizing mechanisms under *t*-CA stress, likely reflecting a more dysregulated cytoplasmic environment.

Notably, genes involved in iron acquisition (including *ent*, *fep*, and *feo* operons) were markedly upregulated in the $\Delta relA$ strain compared to WT (Dataset 1). This effect likely results from the failure to repress siderophore biosynthesis pathways, which are normally downregulated by (p)ppGpp during stress adaptation. These findings align with prior observations (Fig. 3b) and further confirm that virulence-associated iron uptake systems remain active in $\Delta relA$ despite growth inhibition, suggesting incomplete reprogramming of the bacterial virulence machinery. Selected genes expression involved in metabolic processes and virulence traits was then further verified by qPCR analysis in WT and *relA* background and presented on Fig. 4c.

Altogether, these results support the conclusion that *t*-CA-induced antivirulence activity relies not only on metabolic disruption, but also on an intact RelA- and (p)ppGpp-dependent regulatory axis to silence unnecessary or energy consuming functions under stress.

3.7. *t*-CA effectively inhibit biofilm formation

Exposure to *t*-CA markedly impaired biofilm formation and stability in *E. coli*. Scanning electron microscopy evaluation revealed that untreated biofilms of UT189 formed dense, highly structured aggregates embedded in an extensive extracellular matrix, whereas *t*-CA treatment resulted in pronounced biofilm disintegration and predominance of dispersed bacterial cells (Fig. 5a). Consistently, quantitative crystal violet staining demonstrated a significant, dose-dependent reduction in biofilm biomass following *t*-CA exposure at $1 \times$, $0.5 \times$, and $0.25 \times$ MIC compared with untreated controls (Fig. 5b).

At the same time, metabolic activity of biofilm-embedded cells, assessed by the MTT assay, was strongly reduced by *t*-CA, reaching levels comparable to or lower than those observed for conventional antibiotics tested at equivalent relative concentrations (Fig. 5c).

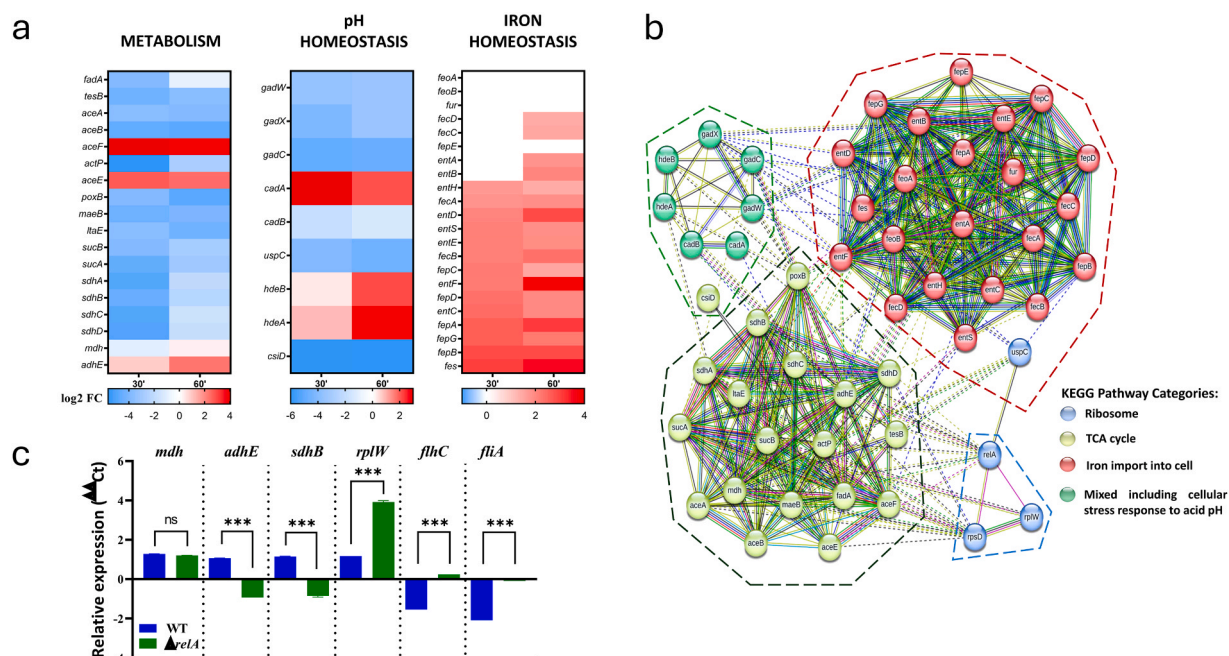


Fig. 4. RelA-dependent transcriptional reprogramming affects key cellular functions (metabolism, pH stress, iron homeostasis) after *t*-CA exposure. (a) Comparative evaluation of transcriptional changes between WT and Δ relA strain. Heatmaps showing log₂ fold change (log₂FC) in gene expression (Δ relA vs. WT) after 30 and 60 min of exposure to 0.25 × MIC *t*-CA. Colour scale represents log₂ fold change relative to WT control. (b) Functional analysis of DEGs assessed by STRING interaction networks. Indicated genes were mapped according to KEGG Pathways categories. (c) Relative expression of selected genes performed by RT-qPCR.

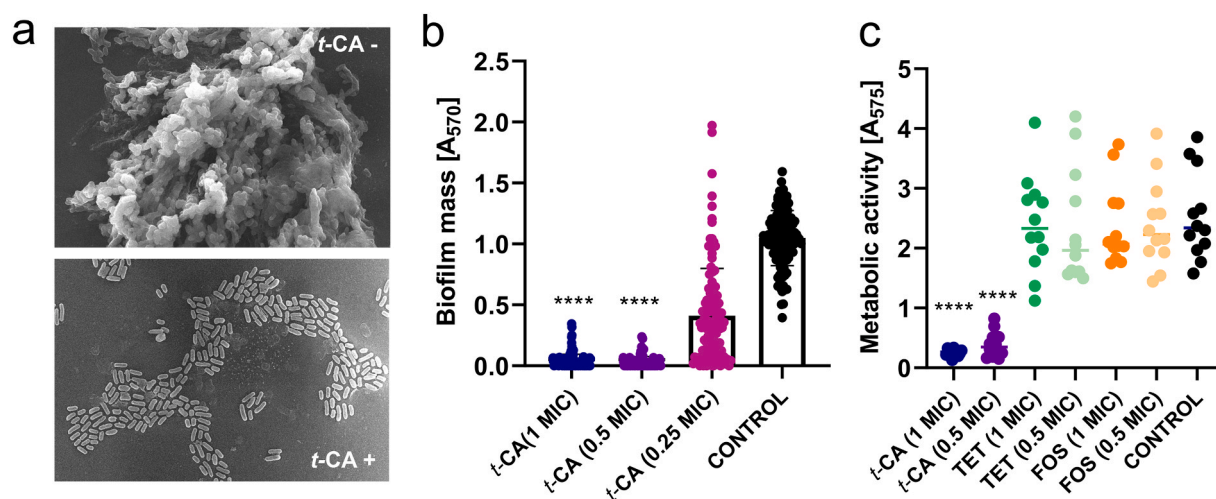


Fig. 5. *t*-CA disrupts biofilm architecture and reduces biofilm biomass and metabolic activity. (a) Scanning electron microscopy (SEM) images of *E. coli* biofilm formed in the absence (*t*-CA-) or presence (*t*-CA+) of trans-cinnamaldehyde, showing pronounced disruption of biofilm structure and reduced cell aggregation upon *t*-CA treatment. (b) Quantitative assessment of biofilm biomass by crystal violet staining (A₅₇₀). *t*-CA treatment was implemented at 1 ×, 0.5 ×, and 0.25 × MIC to starting cultures (UPEC clinical isolates, n = 118). Biofilm mass was assessed spectrophotometrically compared with untreated controls. (c) Metabolic activity of biofilm-embedded cells measured by MTT assay (A₅₇₅) for biofilm producing *E. coli* isolates (n = 12). *t*-CA markedly decreased metabolic activity at inhibitory and sub-inhibitory concentrations, with effects comparable to reference antibiotics tetracycline (TET) and fosfomycin (FOS) at corresponding MIC fractions. Data were collected at least from three independent experiments and shown as individual values for each strain tested, presented with mean ± SD; ****p < 0.0001 versus control.

Complementary phenotypic assays further confirmed that *t*-CA interferes with key biofilm matrix components. Macromorphological analysis of UT189 macrocolonies revealed a pronounced inhibition of curli fibers, cellulose, and phosphoethanolamine-modified cellulose production in the presence of *t*-CA (Figure S3), indicating broad suppression of extracellular matrix assembly. These findings indicate that *t*-CA effectively suppresses both biofilm accumulation and metabolic fitness.

3.8. Cytotoxicity of *t*-CA in epithelial and fibroblast cell lines

In addition, to study the phytochemical safety, the cytotoxic potential of *t*-CA was evaluated in human dermal fibroblasts (HDFa) and human mammary epithelial cells (HB2) following 24 h exposure. As shown in Fig. 6a, both cell lines exhibited a concentration-dependent decrease in viability when treated with increasing concentrations of *t*-CA. Quantitative analysis revealed that HB2 cells were more susceptible to *t*-CA, with an IC₅₀ value of 47.6 μM, whereas HDFa fibroblasts

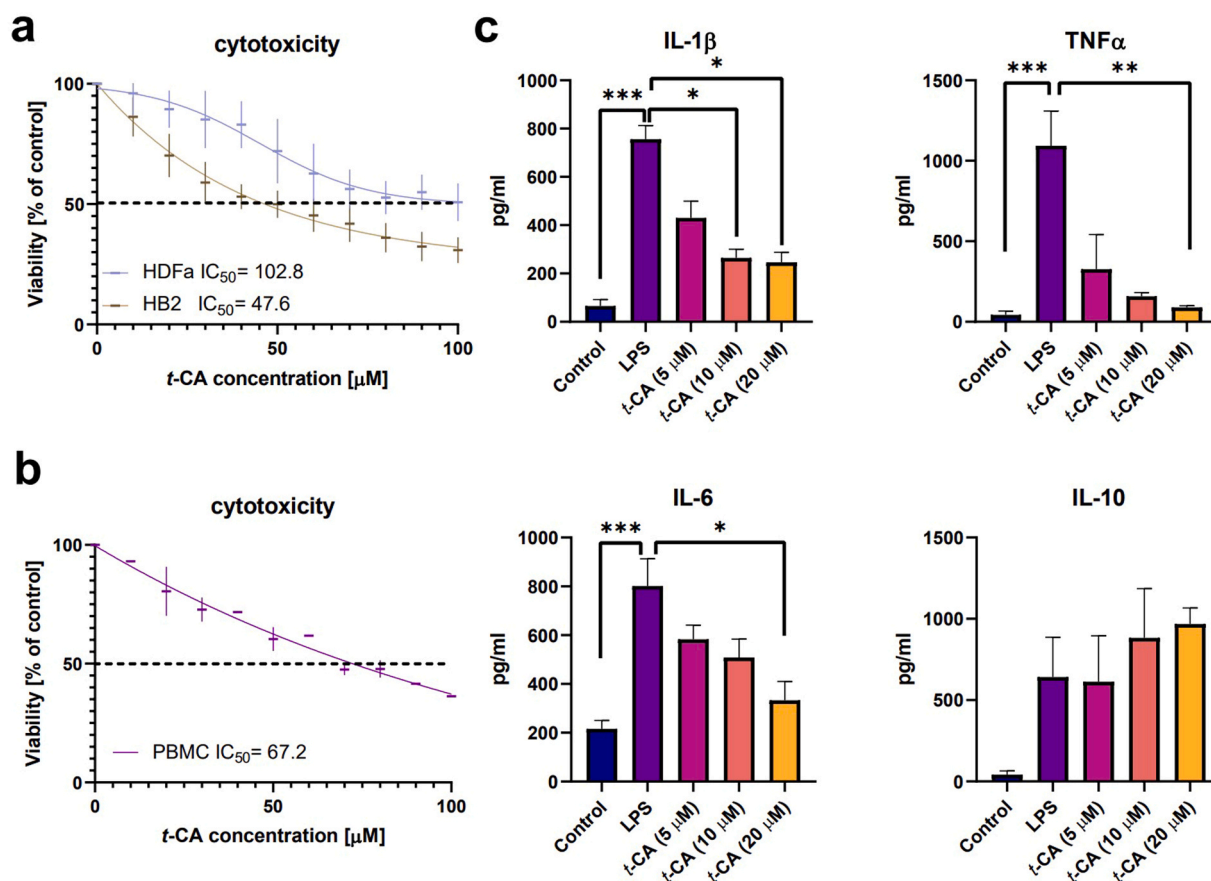


Fig. 6. Cytotoxic and immunomodulatory effects of *t*-CA on mammalian cell lines. (a) Dose-dependent cytotoxicity of *t*-CA in adherent HDFa fibroblasts and HB2 epithelial cells determined by the MTT assay. Cell viability is expressed as a percentage of untreated controls, and IC_{50} values were calculated by nonlinear regression. (b) Cytotoxicity of *t*-CA in peripheral blood mononuclear cells (PBMCs) following 24 h exposure, with the corresponding IC_{50} value indicated. Data are presented as mean $\pm$ SD from at least three independent experiments. The dashed line indicates 50% cell viability. (c) Modulation of cytokine secretion (IL-1 β , TNF- α , IL-6, and IL-10) in LPS-stimulated PBMCs treated with increasing concentrations of *t*-CA (5–20 μ M). Cytokine levels were quantified in culture supernatants by ELISA. Data are presented as mean $\pm$ SD from independent donors (n = 4). Statistical significance for planned comparisons versus LPS is indicated. * p < 0.05, ** p < 0.01, *** p < 0.001.

displayed a higher tolerance with an IC_{50} of 102.8 μ M. Despite the observed differences in sensitivity, neither cell type demonstrated abrupt cytotoxicity within the tested range, suggesting a gradual impact of *t*-CA on cell metabolic activity. Although the concentrations required for antimicrobial efficacy in microbiological assays are considerably higher than the IC_{50} values observed here, *t*-CA itself is a well-characterized phytochemical with an established toxicological and pharmacological profile. These findings, together with its known safety profile, support the view that *t*-CA can be considered relatively safe for applications requiring transient exposure, while still providing potent antiviral activity.

3.9. *t*-CA immunomodulation in PBMC

Next, to further explore the immunological dimension of *t*-CA bioactivity, PBMCs were employed as a physiologically relevant model. *t*-CA markedly modulated cytokine production in LPS-stimulated PBMCs (Fig. 6c). Treatment with *t*-CA resulted in a dose-dependent suppression of key pro-inflammatory mediators, including IL-1 β , TNF- α , and IL-6 with the most pronounced effects observed at the highest concentration tested. In parallel, a trend toward increased secretion of the anti-inflammatory cytokine IL-10 was observed following *t*-CA treatment. This cytokine profile indicates a pronounced immunomodulatory effect of *t*-CA, characterized by attenuation of inflammatory signalling and promotion of a regulatory response. Cytotoxicity assessment in PBMCs revealed an IC_{50} value of 67.2 μ M as determined by trypan blue

exclusion (Fig. 6b). In parallel, viability assays performed in HB2 epithelial cells and HDFa fibroblasts using MTT demonstrated a concentration-dependent reduction in metabolic activity.

3.10. Evaluation of *t*-CA activity in vivo

Finally, the *in vivo* protective effect of *t*-CA was evaluated using the greater wax moth (*G. mellonella*) infection model with the biofilm-forming *E. coli* strain UTI89. Larvae were infected and subsequently treated with increasing doses of *t*-CA to assess post-infection therapeutic potential (Fig. 7). Infected control larvae exhibited rapid and progressive mortality, with survival dropping below 20% within 72–96 h post-infection. In contrast, post-infection administration of *t*-CA significantly improved larval survival in a dose-dependent manner. Treatment with 6.25 and 12.5 mg/kg body weight resulted in a partial but significant delay in mortality, whereas higher doses (25 and 50 mg/kg body weight) conferred the strongest protective effect. At these concentrations, survival rates remained markedly elevated throughout the observation period and were significantly higher than those of untreated infected controls (log-rank test, **** p < 0.0001).

Importantly, *t*-CA treatment did not adversely affect the survival of non-infected larvae, indicating the absence of acute toxicity under the applied dosing regimen. The observed protective effect therefore reflects attenuation of bacterial virulence and/or improved host tolerance, without nonspecific toxicity.

These results demonstrate that *t*-CA retains bioactivity in a complex

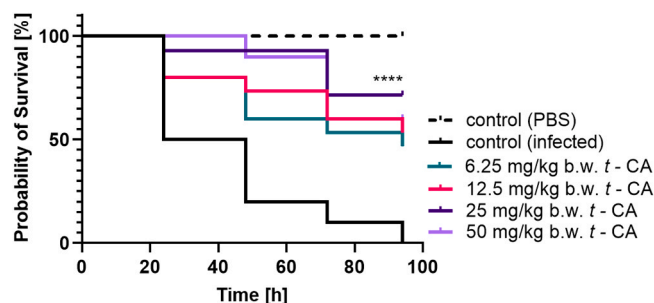


Fig. 7. In vivo activity evaluation of *t*-CA effectiveness in the *G. mellonella* infection model. Kaplan–Meier survival curves of *G. mellonella* larvae infected with *E. coli* UTI89 (10^6 cfu per larvae, pi) and treated post-infection with trans-cinnamaldehyde (*t*-CA). Larvae received *t*-CA at doses of 6.25, 12.5, 25, or 50 mg/kg body weight, and survival was monitored for up to 96 h. Uninfected larvae injected with PBS served as a negative control, while infected but untreated larvae served as the infection control. Statistical significance was assessed using the log-rank against infected control.

host environment and is capable of limiting infection progression when administered post-infection. Together with the transcriptomic and *in vitro* antivirulence data, the *G. mellonella* model provides functional evidence of *in vivo* benefit following post-infection administration of *t*-CA.

4. Discussion

We demonstrated in this study that *t*-CA, a natural phenylpropanoid compound, acts as more than a stressor. Our data support its role in transcriptional changes towards a less virulent phenotype by inducing RelA-dependent reprogramming. These findings position the stringent response and its alarmone, (p)ppGpp, as key modulators of the *E. coli* transcriptional response to *t*-CA phytochemical pressure. While *t*-CA exerts bacteriostatic effects in both WT and $\Delta relA$, indicating that the phytochemical's effect relies not solely on the canonical stringent response, it is worth noting that only in WT cells the virulence-associated processes such as siderophore biosynthesis, quorum sensing, and acid resistance were affected, demonstrating that the antibacterial effects may result from the combined action of (p)ppGpp-mediated metabolism down-regulation and active suppression of virulence genetic traits. Using MG1655 and UTI89 strains as models in the study allowed us to separate a mechanistic regulatory readout from a clinically relevant phenotype. Although strain-specific regulatory wiring may modulate effect sizes, the convergence of transcriptional signatures (virulence/stress pathways) with anti-biofilm phenotypes supports a conserved mode of action.

Notably, there is established consensus that (p)ppGpp is a key regulator of bacterial adaptation and virulence, particularly in the context of host–pathogen interactions [23,24]. The stringent response controls a wide range of processes including secretion systems, motility, biofilm formation, acid tolerance, and nutrient acquisition, enabling pathogens to survive and persist under host-imposed stress [24,25]. In veterinary pathogens such as avian pathogenic *E. coli*, inactivation of *relA* or *spoT* leads to attenuated virulence and reduced colonization, highlighting the importance of stringent response in full pathogenic expression [26]. Our previous studies have shown the potent ability to suppress toxin production and inhibit biofilm formation in *E. coli* and other pathogens using phytochemicals on the route of amino acid starvation conditions governed by RelA synthase [14,27–29]. Exposure to *t*-CA induced significant transcriptional shifts in *E. coli*, but only in the presence of functional *relA* was this accompanied by coordinated downregulation of virulence-associated systems, especially those encoded on mobile genetic elements like toxins encoded by lysogenic bacteriophages [14]. In contrast, in the $\Delta relA$ mutants, the key pathogenic traits were expressed, despite bacterial sensitivity to *t*-CA. This suggests

several possibilities, including the attractive one that the disruption of (p)ppGpp homeostasis can compromise the pathogen's ability to appropriately modulate virulence traits in response to environmental cues. Another one has to incorporate the possibility of more indirect effect of (p)ppGpp deficiency. As the one of most global regulators in the bacterial cell, (p)ppGpp affects both stress response and housekeeping processes such as growth rate control [30]. Therefore, the lack of (p)ppGpp results in the pleiotropic effects. In our experimental set-up, in *relA* mutant strains the secondary regulatory processes may affect the virulence genes' expression. In the multi-facet and multi-level regulatory network with (p)ppGpp as a main controller and supervisor, such a scenario plausible and further studies will be needed to resolve this puzzle.

Our observations suggest that *t*-CA interferes with bacterial metabolism, in part by creating nutrient-limiting or stress-like conditions, such as amino acid starvation, that lead to activation of the stringent response via RelA-mediated (p)ppGpp synthesis. It is important to note that *t*-CA does not directly activate the stringent response; rather, it imposes physiological stress that is sensed by RelA. Induction of pH stress response mechanisms requires substantial pools of endogenous amino acids, such as lysine and serine, as well as metabolic intermediates like pyruvate, to counteract proton imbalance [31,32]. Their rapid depletion may mimic a physiological starvation state, triggering stringent-like adaptation. Additionally, it has been shown that *t*-CA effectively impairs fatty acids synthesis [33], which also trigger RelA dependent stringency control, in *E. coli* [34]. However, in strains lacking RelA, the stress signal remains but cannot be effectively translated into appropriate transcriptional adaptation. This disconnection between stress perception and transcriptional adaptation highlights the role of RelA as a stress sensor and signal transducer for both direct and indirect actions. Hence, the growth-inhibitory effect of *t*-CA was still evident in $\Delta relA$ and $\Delta relA \Delta spoT$ mutants, indicating the existence of RelA-independent control mechanisms (see review [35]). Nonetheless, our current results expand this hypothesis by showing direct transcriptional evidence of *t*-CA-mediated silencing of the systems crucial for bacteria to develop full scale virulence (schematic representation of *t*-CA activity is presented on Fig. 8). It is also worth noting that spontaneous mutations in genes involved in the stringent response, are relatively rare under natural or clinical conditions [36]. This limits the likelihood that resistance to *t*-CA mediated transcriptional modulation would arise through loss or alteration of stringent response function. However, the possibility of occurrence of mutations in genes encoding subunits of RNA polymerase, desensitizing the enzyme to (p)ppGpp, or, in opposite, rendering RNA polymerase status as if (p)ppGpp was always present, cannot be excluded [30,37]. Such mutations may affect the bacterial response to *t*-CA.

One important aspect not directly addressed in this study is the potential interaction between (p)ppGpp-mediated antibiotic tolerance and phytochemical exposure. It has been demonstrated that elevated (p)ppGpp levels associated with *t*-CA effect contribute to transient antibiotic tolerance and persister cells formation in enterohemorrhagic *E. coli* [14]. The role of (p)ppGpp in the persister cells formation and further resuscitation is a matter of many studies and subsequent discussions amongst variety of observed effects and formed hypotheses [38,39]. Given that *t*-CA activates stress response pathways involving RelA, it remains unclear whether this may inadvertently enhance tolerance phenotypes or on the contrary, sensitize bacteria to antibiotic action. While no data to date suggest that *t*-CA promotes stable resistance acquisition, our study did not explore long-term exposure or adaptive responses under combination treatment. It cannot also be excluded that even short-term stringent response induction by *t*-CA may affect the bacterial population response to stress by promoting persisters formation. This effect would need to be further explored. However, on the bright side of the possible *t*-CA effects, several recent reports highlight the synergistic potential of *t*-CA when combined with conventional antibiotics, enabling dose reduction, enhanced clearance, and even

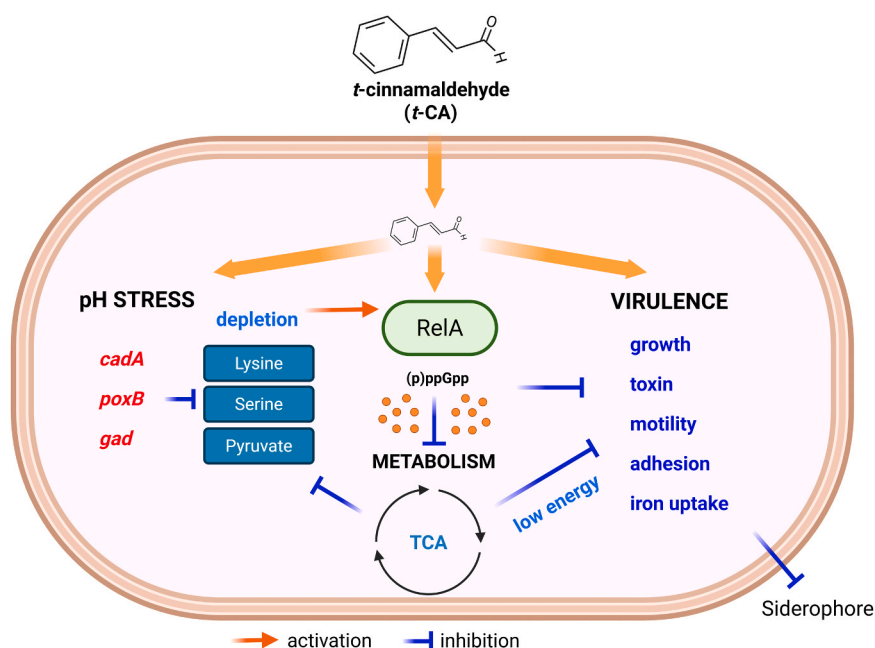


Fig. 8. Proposed model of RelA-dependent modulation of *E. coli* physiology in response to *t*-CA treatment. Exposure to *t*-CA induces nutrient and pH stress, leading to depletion of key metabolites such as lysine, serine, and pyruvate. This activates RelA, triggering synthesis of the stringent response alarmones (p)ppGpp. Elevated (p)ppGpp levels repress central metabolism (TCA cycle) and downregulate multiple virulence-associated pathways, including toxin production, growth, motility, adhesion, and iron uptake. Blue arrows indicate inhibition, while orange arrows indicate activation.

reversal of resistance phenotypes in multidrug-resistant strains [11,28,40]. However, such combinatory effects were not evaluated in the present transcriptomic framework. Future studies should examine whether modulation of the stringent response by *t*-CA influences antibiotic susceptibility, persistence, or synergy in clinically and veterinary-relevant contexts.

From clinical and veterinary perspective, such antivirulence adjuvants are particularly valuable [41,42]. In infections associated with *E. coli* pathogens, including colibacillosis, where iron acquisition, toxin synthesis, and stress resistance are central to pathogenesis, *t*-CA offers a dual advantage: it limits bacterial fitness and simultaneously attenuates key virulence traits. Moreover, its bioactive features enhance the natural barriers of host immunological system [41,42] accompanied by anti-inflammatory properties [43]. Importantly, when used in combination with traditional or narrow-spectrum antimicrobials, *t*-CA has been shown to lower the required dosage, reduce selective pressure for resistance, and improve clinical outcomes [44,45]. *t*-CA is also used as a natural preservative in agricultural products. Recent studies have shown that it can slow bacterial growth and improve shelf life in foods like fresh-cut vegetables and processed meat [46,47]. However, our experimental design relied on short-term *t*-CA exposure at subinhibitory concentration ($0.25 \times \text{MIC}$). More importantly, we used $0.25 \times \text{MIC}$ for RNA-seq analysis to capture early stress signalling and regulatory remodelling without confounding growth arrest, which is a standard approach in antivirulence studies. Although local *t*-CA concentrations in host niches are difficult to estimate due to absorption and matrix binding, transient sub-inhibitory exposures are plausible at mucosal surfaces or during localized delivery where concentration gradients can form. While this mimics realistic phytochemical stress in a host or feed additive context, longer-term adaptation or compensatory mechanisms may not have been captured. Additionally, the impact of *t*-CA in complex host-like environments - such as within gut microbiota, host tissue, or under immune pressure - was not addressed here. However, the cytotoxicity data presented here showed that the phytochemical exhibited moderate or weak cytotoxicity on mammalian cell lines. *t*-CA is classified as a generally recognized as safe (GRAS) substance by the U.S. FDA and is naturally present in cinnamon bark oil, widely used in the food,

cosmetic, and pharmaceutical industries [48]. It occurs as a dietary phytochemical in common human and animal diets, with estimated daily intake from natural sources ranging from 0.1 to 5.0 mg/kg body weight [49]. Toxicological evaluations have shown that oral administration of *t*-CA is well tolerated in mammals, with no observed adverse effect levels (NOAELs) reported up to 250–500 mg/kg bw/day in sub-chronic studies [48]. In our study, post-infection administration of *t*-CA significantly improved larvae survival in a dose-dependent manner, with the strongest protective effect observed at 25–50 mg/kg body weight. Importantly, this *in vivo* benefit is consistent with our previous findings showing that *t*-CA suppresses toxin expression in *E. coli* during interaction with haemocytes in the *Galleria* model [14], supporting an antivirulence component under host-mimicking conditions. At the same time, larval survival alone cannot discriminate between reduced bacterial burden and improved host tolerance, as *E. coli* infection outcomes in *G. mellonella* reflect both pathogen fitness and innate immune responses [50]. Therefore, although our survival data support *in vivo* bioactivity of *t*-CA, bacterial load measurements and virulence-dependency analyses will be required to resolve the relative contributions of bacterial clearance versus host-directed effects.

In summary, this study provides mechanistic insight into how *t*-CA modulates global gene expression in *E. coli* via the RelA-mediated stringent response. It also highlights the therapeutic potential of targeting transcriptional regulation, rather than viability alone, to control bacterial virulence—an approach with significant promise for integrated antimicrobial strategies in human and animal health.

CRediT authorship contribution statement

Patryk Strzelecki: Writing – review & editing, Investigation. **Agnieszka Pyrczak-Felczykowska:** Writing – review & editing, Methodology, Investigation. **Monika Karczewska:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation. **Agnieszka Szalewska-Pałasz:** Writing – review & editing, Supervision, Conceptualization. **Dariusz Nowicki:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Katarzyna Zima: Methodology, Investigation.

Funding

This work was supported by the National Science Centre SONATA grant no. UMO-2018/31/D/NZ7/02258 for D.N., and University of Gdańsk UGrants-start 533-BG10-GS11–25 for M.K.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank all members of the Department of Bacterial Molecular Genetics for their invaluable help, endless discussions, and occasional distractions involving cake and coffee. Without your combined scientific skill, sarcastic commentary, and caffeine-fuelled enthusiasm, this project might still be somewhere between “planned” and “almost started”.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2026.119306](https://doi.org/10.1016/j.biopha.2026.119306).

Data availability

Data will be made available on request.

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Spicing up urinary tract infections: synergistic action of fosfomycin with trans-cinnamaldehyde - insight to the mode of action

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Abbreviations:

AMR, antimicrobial resistance; AU, arbitrary units; CFU, colony-forming units; CLSM, confocal laser scanning microscopy; CV, crystal violet; DCFDA, 2',7'-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; FOS, fosfomycin; GSH, reduced glutathione; MATH, microbial adhesion to hydrocarbons; MBC, minimum bactericidal concentration; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; PBS, phosphate-buffered saline; PDH, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PI, propidium iodide; ROS, reactive oxygen species; SEM, scanning electron microscopy; t-CA, trans-cinnamaldehyde; TPP, thiamine pyrophosphate; UPEC, uropathogenic *Escherichia coli*; UTIs, urinary tract infections; WT, wild type.

Abstract

Objectives: Fosfomycin (FOS) remains an important therapeutic option for urinary tract infections caused by uropathogenic *Escherichia coli* (UPEC), but specific virulence traits as biofilm formation, metabolic adaptation, and antimicrobial resistance may limit its efficacy. This study investigated whether the natural compound, trans-cinnamaldehyde (t-CA) potentiates FOS activity against UPEC and explored the underlying mechanisms of its effect

Methods: The interaction between t-CA and FOS was assessed using checkerboard assays, time-kill analysis. We evaluated biofilm viability and structure using confocal and scanning microscopy as well as catheter-associated biofilm models. Next, effects on membrane integrity, cell-surface properties, membrane potential, intracellular pyruvate levels, and resistance evolution during serial passage were evaluated. Molecular docking was used to explore potential interactions of t-CA with enzymes involved in pyruvate metabolism. *Galleria mellonella* infection model was employed to evaluate *in vivo* therapeutical efficiency.

Results: t-CA potentiated FOS activity against laboratory, reference, and clinical UPEC strains, with synergistic or additive interactions observed across the tested collection. The combination enhanced bacterial killing, reduced biofilm viability and biomass, and disrupted biofilm architecture. In catheter-associated biofilms, combined treatment markedly impaired surface-associated UPEC communities. t-CA reduced extracellular matrix abundance and altered cell-surface hydrophobicity and membrane potential without inducing detectable oxidative stress. Mechanistically, t-CA affected pyruvate homeostasis, reduced intracellular pyruvate levels, and phenotypically intersected with the BtsSR pyruvate-sensing pathway. Serial exposure to FOS alone rapidly increased MIC, whereas t-CA limited this phenomenon and did not itself promote reduced susceptibility. The compounds combination also improved survival of UTI89-infected *G. mellonella* larvae.

Conclusions: t-CA enhances FOS activity against UPEC through complementing the antibiofilm and metabolic effects. By weakening biofilm matrix integrity, perturbing pyruvate homeostasis, and limiting FOS-associated MIC elevation, t-CA represents a promising adjuvant candidate for improving FOS efficacy against biofilm-associated UPEC infections.

Keywords: uropathogens, fosfomycin, pyruvate, antimicrobial, *G. mellonella*

1. Introduction

Antimicrobial resistance (AMR) is accelerating worldwide and increasingly undermines the clinical utility of first-line antibiotics, creating an urgent need for strategies that restore or potentiate antibacterial activity while limiting further resistance selection. Recent surveillance data indicate that resistance is already widespread among pathogens responsible for common community- and hospital-acquired infections, with particularly high rates reported for urinary tract and bloodstream isolates in several regions (WHO, 2025). The long-term outlook remains alarming. Influential global assessments have projected that, without major intervention, AMR could rise to a scale comparable to (or exceeding) cancer mortality by mid-century, reflecting both increasing incidence of hard-to-treat infections and limited replenishment of the antibiotic pipeline.

Urinary tract infections (UTIs) are among the most prevalent bacterial infections and a major driver of antibiotic consumption in outpatient care (WHO, 2025). Uropathogenic *Escherichia coli* (UPEC) dominates uncomplicated UTIs, but treatment is increasingly complicated by resistance to commonly used oral agents. Surveillance programs and regional studies consistently report high resistance rates among outpatient uropathogens to commonly prescribed antibiotics, including trimethoprim–sulfamethoxazole, fluoroquinolones, and β -lactams. In some settings, resistance exceeds 30% for selected drug classes, emphasizing the need for therapeutic strategies that retain activity against resistant and multidrug-resistant isolates. Although UPEC infections are generally associated with low mortality, in otherwise healthy individuals, they have significantly more serious impact on elderly or immunocompromised patients leading to e.g. life threatening urosepsis (Mancuso *et al.*, 2023). For the public health, notably, these strains constitute an important reservoir and vehicle for the dissemination of antimicrobial resistance determinants (Karczewska *et al.*, 2023; Rahman *et al.*, 2022). Moreover, the recurrent nature of UTIs often requires repeated or prolonged antibiotic therapy, resulting in a substantial cumulative economic burden for both patients and healthcare systems (Iskandar *et al.*, 2021; Karczewska *et al.*, 2023).

Fosfomycin (FOS) remains a valuable option in the UTIs treatment strategy (Figure 1A) (Falagas *et al.*, 2010). Clinically, a single 3-g oral dose of fosfomycin trometamol is recommended as a first-line treatment for uncomplicated lower UTIs in women in multiple guidelines and regulatory evaluations (Zheng *et al.*, 2022). Mechanistically, FOS is a phosphoenolpyruvate (PEP) analogue that inhibits the first committed step of peptidoglycan biosynthesis by covalently targeting MurA (UDP-GlcNAc enolpyruvyl transferase), an essential enzyme for cell wall formation. In addition to its broad activity against many uropathogens, including ESBL-producing *E. coli*, FOS benefits from favorable urinary exposure and a comparatively low propensity for widespread resistance in many regions—although resistance can emerge and is often linked to altered uptake (e.g., transporter function) or enzymatic inactivation (Mattioni Marchetti *et al.*, 2023).

A key concept that has gained traction in recent years is that FOS efficacy is tightly coupled to bacterial physiology, particularly metabolic state and transport capacity (Gil-Gil & Martinez, 2022, 2024). Because FOS relies on active transport into the cytoplasm (notably via systems

influenced by central carbon metabolism), metabolic perturbations can alter intracellular drug accumulation and thereby modulate susceptibility. Consistent with this, recent systems-level work demonstrates that metabolic state can shape FOS activity and may even promote the emergence of FOS resistance under certain perturbations, reinforcing the idea that metabolic context is a decisive layer of antibiotic action beyond the canonical target-inhibitor interaction (Peng *et al.*, 2025; Verhulsdonk *et al.*, 2026). This physiology dependence suggests a practical opportunity: adjunct compounds that reprogram metabolism or stress responses may sensitize bacteria to FOS, lowering the effective concentration required for growth inhibition and potentially slowing resistance selection during repeated exposure.

Natural products and plant-derived small molecules represent a valuable source of antibiotic adjuvants because their biological activity often extends beyond direct growth inhibition and includes modulation of stress adaptation, virulence, and central metabolic pathways (Groleau *et al.*, 2026; Strzelecki *et al.*, 2025). Among these compounds, trans-cinnamaldehyde (t-CA, Figure 1B) is particularly relevant due to its ability to affect several interconnected aspects of bacterial physiology. Our previous work demonstrated that t-CA is active against pathogenic *E. coli* and that its antibacterial effect is accompanied by attenuation of virulence-associated processes rather than being explained solely by nonspecific membrane damage (Karczewska *et al.*, 2024). More recent transcriptomic and biochemical analyses showed that sub-inhibitory t-CA concentration induces RelA-dependent accumulation of (p)ppGpp and extensive transcriptional reprogramming, including repression of iron-acquisition systems, quorum-sensing-associated pathways, and acid-resistance mechanisms (Karczewska *et al.*, 2026). These changes were substantially weaker in the absence of RelA, indicating that activation of the stringent response is a central component of the cellular response to t-CA. In parallel, t-CA impaired extracellular matrix production and reduced biofilm biomass and metabolic activity, confirming that its effects extend to physiologically heterogeneous bacterial populations and affects global metabolic pathways. Thus, t-CA should not be considered merely as a membrane-active phytochemical, but rather as a stress- and metabolism-modulating compound capable of altering the physiological state that determines antibiotic susceptibility. This multi-target activity provides a mechanistic basis for testing t-CA as an adjuvant to FOS, whose uptake and bactericidal activity are strongly influenced by bacterial metabolic status and active cell-wall synthesis. We therefore reasoned that t-CA-mediated disruption of stress adaptation and metabolic homeostasis could increase the susceptibility of *E. coli* to FOS and reduce the concentrations required for effective growth inhibition.

In this study, we investigated whether combining FOS with t-CA enhances inhibitory activity against *E. coli* and whether such enhancement is consistent with a metabolism-linked interaction. We hypothesized that these agents can act synergistically through interconnected physiological pathways whereby t-CA-driven metabolic and/or stress perturbations sensitize *E. coli* to MurA inhibition by FOS leading to stronger growth suppression at reduced concentrations compared with monotherapy. In addition, given the known propensity of bacterial populations to adapt under sustained antibiotic pressure, we examined whether the combination influences the trajectory of susceptibility during serial exposure, a feature that

would further support the value of metabolic adjuvants as resistance-mitigating partners for existing first-line drugs.

2. Materials and methods

2.1 Bacterial strains and growing conditions

Bacterial strains used in this study are listed in Table X. Strains were maintained as glycerol stocks at -80°C and, prior to each experiment, were streaked onto solid agar (LB Miller, Sigma) plates and incubated under the appropriate conditions, typically at 37°C . Where indicated, strains were grown on Mueller–Hinton II (MH II, Merck) or YESCA broth (1 g/L yeast extract and 10 g/L casamino acids). Single colonies were selected to initiate overnight cultures in the relevant broth medium and then used for the appropriate assays. Where indicated, cells were washed with sterile buffered saline (PBS).

2.2 Susceptibility testing

Susceptibility testing was conducted according to standard CLSI methods (CLSI, 2018). Briefly, the microdilution assay was employed to establish minimum inhibitory concentrations (MICs) of the tested compounds against indicated bacterial strains. The interaction between the antibiotics and *t*-CA was subsequently evaluated using the checkerboard method to determine the fractional inhibitory concentration index (FICI). The assay was performed in 96-well microtiter plates using the previously determined MIC values as a baseline. Stock solutions of the compounds were prepared at 16 times the respective MIC, followed by serial two-fold dilutions of each drug. The first compound was diluted along the ordinate (rows), while the second was diluted along the abscissa (columns), after which the solutions were combined. Each microtiter well was then inoculated with 100 μL of bacterial inoculum to achieve a final concentration of 5×10^5 CFU/mL at 200 μL of broth. The plates were incubated at 37°C for 20 hours, and the absorbance at 570 nm was measured using an EnSpire plate reader (PerkinElmer, Singapore). FICI was calculated as:

$$\text{FICI} = \frac{\text{MIC}_A \text{ (in combination)}}{\text{MIC}_A \text{ (alone)}} + \frac{\text{MIC}_B \text{ (in combination)}}{\text{MIC}_B \text{ (alone)}}$$

Interactions were interpreted as synergistic ($\text{FICI} \leq 0.5$), additive ($0.5 < \text{FICI} \leq 1$), indifferent ($1 < \text{FICI} \leq 4$), or antagonistic ($\text{FICI} > 4$)

Bacterial kill kinetics were assessed using a time-kill assay (Klepser *et al.*, 1998). Briefly, a fresh bacterial culture was adjusted to 0.5 McFarland and diluted in PBS to an initial inoculum of approximately 1×10^5 CFU/mL. The suspension was exposed to the indicated antimicrobial agents at the specified concentrations and incubated at 37°C . At indicated time points, aliquots were withdrawn, serially diluted in sterile PBS, and plated on agar for CFU enumeration after overnight incubation at 37°C . Bactericidal activity was expressed as $\log^{10}(\text{CFU/mL})$ over time and presented as time-kill curves.

2.3 Live/dead flow cytometry analysis

Live/dead analysis was performed using a two-color fluorescence assay (Thermo Fisher Scientific; L7012) (Konieczny *et al.*, 2021). Overnight cultures of *E. coli* strains were prepared and adjusted to approximately 0.5 McFarland. Cells were then exposed to t-CA and fosfomycin at the indicated concentrations and incubated at 37 °C by 120min. Following treatment, cells were stained with a SYTO dye and propidium iodide (PI). SYTO labels all cells, whereas PI penetrates only cells with compromised membranes, enabling discrimination of live (SYTO-positive/PI-negative) and membrane-compromised/dead (PI-positive) populations. Samples were acquired on an Amnis FlowSight imaging flow cytometer (Luminex). Single cells were gated based on brightfield features to exclude aggregates and debris. Fluorescence compensation was performed using single-stained controls. SYTO fluorescence was excited with the 488 nm laser and collected in the green channel, whereas PI fluorescence was collected in the red channel. Data were analyzed using IDEAS (Luminex) and FlowJo. Live and dead populations were quantified using fluorescence thresholds defined from appropriate controls.

2.4 Antimicrobial activity evaluation in biofilms

The catheter biofilm assay was performed based on Borowicz *et al.* with minor modifications (Borowicz *et al.*, 2023). Bacteria were grown overnight at 25 °C in YESCA medium (1 g/L yeast extract and 10 g/L casamino acids) with shaking (120 rpm), adjusted to 4 McFarland units, and diluted 1:100 in fresh YESCA. Sterile Nelaton catheters (Unomedical, UK) were cut into 10 cm segments and connected to sterile syringes. The bacterial suspension was drawn into each catheter-syringe assembly (final volume $\leq$ 3 mL) and sealed aseptically. Catheters were incubated statically at 25 °C for 72 h to allow biofilm development. To compare antibiofilm activity, catheters were incubated with: (i) inoculum without additives (positive biofilm control), (ii) inoculum supplemented with FOS at the indicated concentration, (iii) inoculum supplemented with t-CA at the indicated concentration, and a combined-treatment condition (FOS+t-CA; MIX) was included. A sterility control consisted of catheters filled with sterile medium only. After incubation, catheters were opened and gently rinsed with 20 mL distilled water to remove planktonic cells. Biofilm biomass was quantified by staining with 1% (w/v) crystal violet for 20 min at room temperature, rinsing until the wash was clear, photographing the catheters, and solubilizing the bound dye with 2 mL of 33% (v/v) acetic acid; absorbance was measured at 570 nm (BioSpectrometer, Eppendorf, Germany). In parallel, biofilm viability was assessed using an MTT reduction assay: after rinsing, catheter segments were incubated with MTT in fresh medium in the dark, the resulting formazan was dissolved in an organic solvent, and absorbance was measured spectrophotometrically (typically at 570 nm) and expressed relative to the untreated biofilm control after background subtraction (sterility control).

2.5 Microscopy techniques evaluation

Fluorescence staining was routinely assessed by fluorescence microscopy using Leica DMI4000B inverted microscope fitted with a DFC365FX camera (Leica Microsystems, Germany). Scanning electron microscopy (SEM) was employed for imaging of biofilm structure morphology in medical devices. Catheter segments with biofilm were aseptically cut

and gently rinsed with sterile PBS to remove loosely attached cells and debris. Samples were fixed in 2.5% (v/v) glutaraldehyde in PBS for 1–2 h at room temperature under gentle agitation, then rinsed three times with PBS. Post-fixation was performed in 1% (w/v) osmium tetroxide (OsO₄) for 1–2 h at room temperature, followed by three rinses with sterile distilled water. Specimens were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100% ethanol; 10–15 min each step), using fresh ethanol for each step and ensuring complete dehydration. Samples were then dried by critical point drying (CPD), mounted onto aluminum SEM stubs, and sputter-coated with gold using a Spi Module Sputter Coater. Imaging was performed using a Philips XL-30 (FEI) scanning electron microscope (Laboratory of Bioimaging, University of Gdańsk, Gdańsk, Poland) operated at an accelerating voltage of 5 or 15 kV.

Confocal laser scanning microscopy (CLSM). Biofilms of UTI89 were grown in LabTek™ chamber slides as described in Sect. 2.8. After treatment (FOS 2×MIC, t-CA 2×MIC, or combination: FOS 1×MIC + t-CA 1×MIC) and washing, biofilms were stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific; L7012). The staining solution was prepared by adding 1.5 µL SYTO 9 and 1.5 µL propidium iodide (PI) to 1 mL saline, followed by incubation at room temperature for 20 min protected from light. The staining mixture was removed, chambers were detached from the slide, and samples were covered with a coverslip and mounted using low-melting agarose. Bacterial biofilms were imaged using a Leica Stellaris 5 confocal laser scanning microscope controlled with LAS X software. Image stacks were acquired in xyz mode using an HC PL FLUOTAR L 40×/0.60 dry objective, with a zoom factor of 1. The pinhole was set to 1 Airy unit. Line averaging was set to 3, while frame averaging, frame accumulation and line accumulation were set to 1. Excitation was provided by a white light laser (WLL), and two acquisition settings were used. In the first setting, excitation was performed at 495 nm with a laser line intensity of 2.29%, and detector gain and offset were set to 25.8 and 0, respectively. In the second setting, excitation was performed at 549 nm with a laser line intensity of 5.41%, and detector gain and offset were set to 63.5 and 0, respectively. For each analysed biofilm, confocal z-stacks consisting of nine optical sections were acquired. Orthogonal views were generated from the original z-stacks. The z-stack datasets were subsequently transferred to LAS AF software for three-dimensional visualisation and reconstruction. Comparable samples were imaged using the same acquisition settings.

2.6 Resistance evolution assay

Evolution experiments were performed by serial passaging under antibiotic selection. Overnight cultures were initiated from fresh grown single colonies and resuspended to OD₆₀₀ = 0.005. Cultures were used to inoculate 96-well plates containing LB and a two-fold serial dilution gradient of fosfomycin (with or without t-CA at the indicated fixed concentration, where applicable) and incubated for 24 h at 37 °C with agitation. Optical density was measured using an EnSpire microplate reader. Wells were considered to show growth if OD reached at least 50% of the no-drug LB control. For each passage, the culture growing at the highest fosfomycin concentration was transferred into fresh LB containing the corresponding two-fold fosfomycin gradient (± t-CA) and adjusted again to OD₆₀₀ = 0.005. Five serial passages were performed unless resistance reached the highest fosfomycin concentration soluble in LB. In

control experiments performed with t-CA alone (at the same fixed concentration as in combination assays), no increase in tolerance was observed under the applied conditions. Experiment was done at least three times in triplicates

2.8 Molecular docking

Molecular docking of t-CA was performed using CB-Dock2 with automatic pocket detection and docking (Liu *et al.*, 2022; Yang *et al.*, 2022). The t-CA ligand (CAS: 14371-10-9) was provided as a 3D structure file (MolFile). Receptor structures were obtained from the Protein Data Bank: AceE (PDH E1; PDB ID: 2IEA), AceF (PDH E2; PDB ID: 8OSY), LpdA (PDH E3; PDB ID: 4JQ9), and PoxB (PDB ID: 3EYA). Prior to docking, co-crystallized ligands and all crystallographic water molecules were removed from each receptor structure. Docking poses were ranked according to the CB-Dock2 scoring output. Protein-ligand interactions and 2D contact residues were analyzed and illustrated using BIOVIA Discovery Studio Visualizer. Docking poses were prioritized based on their localization within the predicted binding pockets corresponding to catalytically relevant regions (active-site) of each target, as inferred from pocket placement relative to functionally important residues and the overall interaction pattern (hydrogen bonding, π -interactions, and hydrophobic contacts).

2.9 Cell-surface alteration evaluation

Cell surface hydrophobicity was assessed by using the MATH assay (Pembrey *et al.*, 1999). Briefly, overnight bacterial cultures were refreshed and grown to an optical density of OD = 0.2. Cultures were then exposed to t-CA, FOS, or their combination (MIX), while untreated cells served as the control. Samples were incubated with shaking, and aliquots were collected after 30 and 60 min of treatment. The absorbance of each bacterial suspension was measured at 550 nm and recorded as A_0 . Xylene was then added to each sample, and the mixtures were vigorously vortexed to allow interaction between bacterial cells and the hydrocarbon phase. Samples were left undisturbed until complete phase separation. The lower aqueous phase was carefully collected, and its absorbance was measured again at 550 nm and recorded as A_1 . Cell-surface hydrophobicity was calculated as the percentage of cells adhering to xylene according to the formula:

$$\text{Hydrophobicity (\%)} = [(A_0 - A_1) / A_0] \times 100.$$

Membrane potential effects were conducted using the voltage-sensitive dye 3,3'-dipropylthiadicarbocyanine iodide [DiSC₃(5)] (Buttress *et al.*, 2022). Bacterial cultures were grown to an optical density of OD = 0.5 and centrifuged for 15 min at 4000 rpm. The cell pellet was resuspended in PBS containing 1% DMSO. The cell suspension was transferred to wells of a black microplate and mixed with DiSC₃(5), prepared in DMSO, to a final concentration of 1 μ M. Samples were incubated for 25 min at 37°C in the dark to allow dye equilibration. Fluorescence was then monitored at an excitation wavelength of 652 nm and an emission wavelength of 671 nm at 1-min intervals until a stable baseline signal was obtained. After baseline stabilization, the tested compounds were added, and fluorescence measurement was continued at 1-min intervals. Increased fluorescence was interpreted as a change in membrane potential consistent with membrane depolarization.

2.10 Antimicrobial efficiency of the antimicrobial agents in *G. mellonella* *in vivo* model

Greater wax moth larvae *Galleria mellonella* L. (Lepidoptera: Pyralidae) (Exoticroom, Łódź, Poland) were stored in the dark at 20 °C until use. Healthy larvae weighing approximately 250–300 mg, without signs of melanization or visible dark spots on the cuticle, were selected for experiments (Budziaszek *et al.*, 2023). Larvae were infected with *E. coli* UTI89 at a dose of 10⁵ CFU per larva using a Hamilton syringe fitted with a blunt-ended needle. The bacterial suspension (10 µL) was injected into the last left proleg. One hour post-infection, larvae received a second injection (10 µL) into the last right proleg containing either t-CA and FOS in combination or each compound alone. Control larvae were injected with phosphate-buffered saline (PBS). Following treatment, larvae were placed in Petri dishes lined with Whatman filter paper and incubated at 37 °C in the dark. Survival and health status were monitored every 24 h for 4 consecutive days, with morbidity assessed by changes in behavior and melanization. Larvae were considered dead upon lack of movement in response to touch and the presence of extensive dark pigmentation. Each treatment group contained 15 larvae, and all experiments were performed at least three independent times.

2.11 Statistic methods

All experiments were performed using at least three independent biological replicates, unless stated otherwise. Data are presented as the mean ± standard deviation (SD), as specified in the corresponding figure legends. Comparisons between two groups were performed using an unpaired, two-tailed Student's t-test. Comparisons involving three or more groups were analyzed by one-way analysis of variance (ANOVA), followed by an appropriate multiple-comparisons post hoc test, as indicated in the figure legends. Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results

Susceptibility and interactions of antimicrobial compounds

To identify antibiotic combinations capable of enhancing the antibacterial activity of t-CA, we first screened its interactions with representatives of several antibiotic classes using the checkerboard assay (Figure S1). Among the tested compounds, FOS produced the most pronounced and reproducible synergistic interaction with t-CA (Figure 1C), whereas the remaining antibiotics showed predominantly additive, or even adverse effect, observed for oxytetracycline.

STRAIN	<i>Trans</i> -cinnamaldehyde [mg/l]			Fosfomycin [mg/l]			FICI	Interaction
	One-component	Combination	Fractional Inhibitory Concentration [FIC]	One-component	Combination	Fractional Inhibitory Concentration [FIC]		
MG1655	500	62.5	0.125	4	1	0.25	0.375	synergistic
UTI89	500	16	0.03125	4	1	0.25	0.28125	synergistic
CFT073	500	125	0.25	4	1	0.25	0.5	synergistic
KB077	500	62.5	0.125	4	1	0.25	0.375	synergistic
KB075	500	31	0.0625	2	1	0.5	0.5625	additive
KB076	500	125	0.25	2	0.5	0.25	0.5	synergistic
KB082	500	31	0.0625	4	1	0.25	0.3125	synergistic
KB083	500	16	0.03125	4	2	0.5	0.53125	additive
KB097	500	125	0.25	4	0.5	0.125	0.375	synergistic
KB047	500	62.5	0.125	4	1	0.25	0.375	synergistic

Table 1. Interaction between t-CA and FOS against laboratory, reference *E. coli*, and clinical UPEC strains. MIC values of t-CA and FOS applied individually and in combination were determined using the checkerboard microdilution assay.

To determine whether t-CA enhances the antibacterial activity of FOS, their interaction was evaluated against the laboratory strain MG1655, the reference UPEC strains UTI89 and CFT073, and seven clinical *E. coli* isolates. The MIC of t-CA alone was 500 mg/L for all tested strains, whereas the MIC of FOS alone ranged from 2 to 4 mg/L. In combination, the effective concentrations of both compounds were substantially reduced.

Synergistic interactions were observed for eight of the ten tested strains, with FICI values ranging from 0.28125 to 0.500. The strongest interaction was detected for UTI89, for which the effective concentration of t-CA decreased from 500 to 16 mg/L and that of FOS from 4 to 1 mg/L, resulting in a FICI of 0.28125. This corresponded to approximately a 31-fold reduction in the effective t-CA concentration and a four-fold reduction in the effective FOS concentration compared with the respective monotherapies. A similarly strong interaction was observed for KB082, with a FICI of 0.3125.

Checkerboard survival maps further demonstrated that the combinations associated with FICI values below 0.5 coincided with marked reductions in bacterial survival (Figure 2A). The strongest reduction was observed when both compounds were used at fractional concentrations below their respective MICs, indicating that the interaction was not explained by the independent activity of either compound alone.

The synergistic effect was further validated in time-kill experiments (Figure 2B). In all three strains, the combination produced a faster and more extensive decline in viable cell counts than either monotherapy. In UTI89 and CFT073, treatment with FOS or t-CA alone resulted only in partial reductions in CFU/mL, whereas the combination decreased bacterial counts to the

detection limit after 180 min. A similar effect was observed for MG1655, although t-CA alone displayed stronger intrinsic activity against this strain than against the UPEC isolates. Nevertheless, the combination was consistently more bactericidal than FOS alone and was required for complete killing of the tested UPEC and wild-type strains under the applied conditions.

The effect of the combination on membrane integrity was examined using SYTO/PI staining followed by imaging flow cytometry (Figure S2, 2C). Treatment with the combination caused a marked shift from the live-cell population toward the PI-positive fraction. Approximately 86.7% of cells were classified as membrane-compromised or dead, whereas only 10.2% retained the fluorescence profile characteristic of viable cells. The distribution of the treated population partially overlapped with that of the 70% ethanol control and was clearly separated from the untreated control. These results indicate that the enhanced bactericidal activity of the t-CA/FOS combination is accompanied by extensive loss of membrane integrity.

Combining the t-CA and FOS agents reduces biofilm viability and disrupts its architecture.

The effect of t-CA/FOS combination on biofilm formation was first screened in a 96-well plate model (Figure 3). Fluorescence imaging after SYTO 9/PI staining showed that untreated biofilms were dominated by SYTO 9-positive cells, whereas exposure to either t-CA or FOS increased the proportion of PI-positive cells. The most pronounced shift toward the PI-positive population was observed following treatment with the combination, indicating extensive membrane damage and loss of viability within the biofilm (Figure 3A). This observation was confirmed by the MTT assay. Both compounds applied individually reduced the metabolic activity of biofilm-associated cells compared with the untreated control, although the magnitude of the effect differed between strains (Figure 3B). In all three tested strains, the combination produced the strongest decrease in MTT reduction. The reduction was particularly marked for MG1655 and CFT073, while a similar tendency was observed for UTI89. These results indicate that the combined treatment impairs biofilm-associated cell viability more effectively than either compound used alone.

The structural effects of the treatment were subsequently examined by confocal microscopy and in a catheter-associated biofilm model (Figure 4). Confocal imaging showed that FOS and t-CA reduced biofilm continuity and increased the proportion of PI-positive cells, while the combination caused the most pronounced disruption of three-dimensional organization, yielding a thinner and highly fragmented biofilm dominated by membrane-compromised cells (Figure 4A). These changes were evident relative to the untreated control, which formed a dense and predominantly viable biofilm. Even more pronounced pattern was observed on catheter surfaces (Figure 4B). FOS treatment reduced bacterial accumulation and the overall extent of surface colonization, whereas t-CA caused a pronounced depletion of the matrix surrounding the cells and disrupted the cohesion of bacterial aggregates. The combination further enhanced both effects, producing the lowest bacterial coverage and the most substantial loss of matrix-associated material. Quantitative measurements confirmed that the combined treatment caused the greatest reduction in both biofilm viability and total biomass (Figure 4C).

t-CA alters the properties of the cell surface and membrane polarization without inducing ROS.

Next, to provide mechanistic insight into the biofilm-associated phenotypes observed above, we investigated whether the reduced viability of biofilm cells was linked to oxidative stress and whether the alterations in extracellular matrix organization were accompanied by changes in cell-surface properties. We therefore assessed intracellular ROS production, cell-surface hydrophobicity, and membrane polarization following exposure to t-CA, FOS, and their combination.

Intracellular ROS production was assessed using DCFDA (Figure 5A). None of the tested concentrations of t-CA, FOS, or their combinations increased fluorescence relative to the untreated control, whereas H₂O₂ produced a pronounced signal. These results indicate that the antibacterial activity of t-CA and the t-CA–FOS combination was not associated with detectable oxidative stress under the tested conditions.

Cell-surface hydrophobicity was evaluated using the microbial adhesion to hydrocarbons (MATH) assay (Figure 5B). Exposure to t-CA significantly reduced surface hydrophobicity compared with the untreated control, while FOS alone had no detectable effect. A similar reduction was observed for the combination, indicating that the change in surface properties was primarily attributable to t-CA. This finding is consistent with the biofilm-associated alterations observed previously and suggests that t-CA affects bacterial surface features involved in adhesion and biofilm organization.

Membrane polarization was monitored using the potential-sensitive dye DiSC₃(5) (Figure 5C). t-CA induced a gradual increase in fluorescence, consistent with partial membrane depolarization, whereas FOS alone produced only a minor change relative to untreated cells. The combination caused a stronger fluorescence increase than t-CA alone, suggesting an enhanced disturbance of membrane potential. Colistin B, used as a positive control, produced the most pronounced and rapid depolarization.

t-CA targets the pyruvate node and induces compensatory metabolic alterations

Previous work from our group indicated that t-CA affects central carbon metabolism in *E. coli*, including the upregulation of *poxB*, which encodes pyruvate oxidase (Karczewska *et al.*, 2024). Because FOS activity is influenced by the physiological and metabolic state of the cell, including pathways associated with pyruvate utilization, we investigated whether t-CA could interact with enzymes operating at the pyruvate node. Molecular docking was therefore performed against PoxB and three catalytic components of the pyruvate dehydrogenase complex: AceE, AceF, and LpdA (Figure 6.)

Docking predicted energetically favorable poses for all four targets, with calculated binding energies ranging from −5.8 to −6.7 kcal/mol (Table 2). The lowest docking score was obtained for AceF, followed by PoxB, LpdA, and AceE. However, the biological relevance of the predicted interactions differed between the proteins. In AceE, t-CA was positioned within the TPP-binding cavity, adjacent to the catalytic region, suggesting that ligand binding at this site could interfere with cofactor positioning, substrate access, or local conformational dynamics.

Although the predicted energy for AceE was less favorable than that obtained for AceF, the location of the binding pose provides a stronger mechanistic rationale for a potential effect on PDH activity. In contrast, the PoxB-binding cavity was substantially larger and accommodated numerous surrounding residues, making the predicted interaction less indicative of a specific inhibitory pose. Thus, the PoxB docking result was considered supportive of possible association but insufficient to infer direct inhibition.

Target	kcal/mol	AAs interactions	Cavity volume (Å ³)	Contact residues
PoxB	-6.4	6	4895	Chain A: THR25 GLY26 ASP27 GLU50 SER73 CYS74 PRO76 GLY77 HIS80 PHE112 GLN113 Chain B: ASN83 HIS92 GLY209 GLY211 ALA233 LEU234 ARG235 THR251 GLY252 LEU253 ILE254 GLY255 GLY273 THR274 GLN275 PHE276 PRO277 TYR278 ILE291 ASP292 ILE293 ASN294 SER297 GLY310 ASP311 ILE312 VAL380 GLY381 THR384 VAL385 SER402 PHE403 ASN404 GLY406 SER407 MET408 GLY432 ASP433 GLY434 GLY435 MET438 ASN460 VAL462 LEU463 GLY464 PHE465 VAL466
AceE (PDH E1)	-5.8	8	1776	Chain A: HIS106 SER109 SER112 GLN140 HIS142 TYR177 VAL192 SER193 MET194 GLY229 ASP230 GLY231 GLU232 GLU235 ASN258 ASN260 GLN262 ARG263 LEU264 LYS392 ASP609 Chain B: ALA520 ASP521 GLU522 ILE569 GLU571 TYR599 PHE602 ARG606 GLU636 HIS640
AceF (PDH E2)	-6.7	3	2601	Chain A: PHE428 ASP429 LYS430 THR558 HIS559 ALA578 MET579 GLU580 PRO581 VAL582 TRP583 MET593 Chain B: PHE428 LYS430 THR558 HIS559 ALA578 MET579 GLU580 PRO581 VAL582 TRP583 MET593 Chain C: PHE428 LYS430 THR558 HIS559 LYS576 SER577 ALA578 MET579 GLU580 PRO581 PRO595
LpdA (PDH E3)	-6.1	5	1424	Chain A: ALA382 ALA383 SER384 GLY385 ILE388 GLU436 ALA439 LEU440 ILE442 HIS445 GLU460 SER462 ASP465 LEU466 PRO467 Chain B: PRO16 TYR19 ARG24 GLY42 CYS45 LEU46 ILE51 GLN96 LEU97 GLY100 LEU101 MET104 HIS326 HIS329 VAL330 GLU333 TYR341 PHE342 ASP343 PRO344

Table 2. Predicted interactions of t-CA with enzymes involved in pyruvate metabolism in *E. coli*. Docking scores and cavity volumes were obtained using CB-Dock2, while ligand–protein contacts were identified with BIOVIA Discovery Studio Visualizer.

To determine whether these predicted interactions were accompanied by changes in pyruvate metabolism, intracellular pyruvate levels were measured following exposure to subinhibitory t-CA concentrations (Figure 7A). A significant decrease in the intracellular pyruvate pool was observed in cells treated with t-CA alone and with the t-CA/FOS combination. This finding argues against simple pyruvate accumulation and instead indicates altered pyruvate turnover or redistribution of carbon flux. One possible model is that partial perturbation of AceE-dependent PDH activity induces a compensatory metabolic response involving PoxB. Increased PoxB activity would provide an alternative route for direct conversion of pyruvate to acetate and CO₂, potentially lowering the steady-state pyruvate pool while partially bypassing impaired PDH-dependent flux.

This interpretation is consistent with our previous observation that t-CA induces *poxB* expression without corresponding changes in overflow pathway (Pta-AckA) (Karczewska *et al.*, 2024). Such a transcriptional pattern is more compatible with activation of the direct PoxB-dependent route than with classical acetate overflow through the Pta-AckA pathway. Increased conversion of pyruvate to acetate may also explain the previously observed engagement of the lysine decarboxylase-dependent acid stress response. Acetate production and extracellular acidification could activate the Lysine decarboxylation system, which counteracts low-pH stress through proton-consuming lysine decarboxylation. Thus, to further evaluate whether t-CA affects pathways linked to pyruvate metabolism and acid-stress adaptation, spot assays were performed using deletion mutants impaired in metabolic functions selected for the previous test (Figure 7B). Under control conditions, all tested strains retained growth across serial dilutions, although strain-dependent differences in colony density were visible. Exposure to subinhibitory concentrations of t-CA revealed a clear phenotype in mutants defective in PDH components. In particular, disruption of *aceE* and *aceF* markedly impaired growth in the presence of t-CA, indicating that an already compromised PDH complex strongly sensitizes *E. coli* to t-CA exposure (Figures 7B). This phenotype was subsequently confirmed in the UPEC strain UTI89 and its isogenic PDH mutants (Figure S3). This was particularly evident for strains defective in *aceE*, *aceF*, or *lpdA*, supporting the conclusion that PDH-linked pyruvate metabolism contributes to tolerance of t-CA/FOS-mediated stress in a clinically relevant UPEC background.

In contrast, deletion of *ldcC* or *poxB* did not increase susceptibility to t-CA. The growth of these mutants remained broadly comparable to that of the WT strain under the tested conditions. This suggests that LdcC-dependent lysine decarboxylation and PoxB-dependent pyruvate oxidation are unlikely to represent primary cellular targets of t-CA. Rather, these pathways may function as downstream or compensatory responses activated during t-CA-induced metabolic stress.

Additional support for the involvement of pyruvate homeostasis was obtained using strains differing in the BtsSR pyruvate-sensing system (Figure 7C). The contribution of BtsSR to the t-CA-induced phenotype was examined employing *btsSR*-deficient strains. In the presence of a functional BtsSR system, both t-CA (0.125 x MIC) and exogenous pyruvate (60μM) abolished

Congo red binding, consistent with reduced production of matrix-associated components. This response was not observed in the *btsSR*-deficient background, which retained the Congo red-positive phenotype under both treatment conditions. Thus, the effects of t-CA and pyruvate on biofilm matrix production were dependent on BtsSR-mediated pyruvate sensing.

The t-CA/FOS combination attenuates the development of fosfomycin resistance

The emergence of antimicrobial resistance is a major concern in the treatment of recurrent UTIs, where repeated antibiotic exposure may promote the selection of less susceptible bacterial populations (Figure 8). Serial exposure to FOS alone resulted in a rapid and progressive increase in MIC, reaching approximately 300-fold above the initial value by day 5. The addition of t-CA delayed this increase in a concentration-dependent manner, with 0.25×MIC t-CA providing the strongest suppression of development of FOS resistance. In contrast, repeated exposure to t-CA alone did not result in a detectable increase in MIC or reduced susceptibility. Thus, the finding that, t-CA limited the development of reduced susceptibility to FOS without itself promoting resistance during serial passage was both unexpected and intriguing. We next asked whether t-CA could improve the activity of FOS against FOS-resistant UPEC strains with different resistance backgrounds (Figure 8B). The combination showed no relevant interaction in strains carrying FosA-type metalloenzymes, including R82 (*fosA3*) and R4880 (*fosA4*), indicating that t-CA did not restore FOS activity when resistance was mediated by enzymatic drug inactivation. In contrast, the FOS-resistant strain N-279, which did not carry a detectable FosA-type determinant, responded positively to the combination. In this strain, the presence of t-CA reduced the effective FOS concentration fourfold, from an initial MIC of 2 g/L to 0.5 g/L.

The t-CA/FOS combination treatment improves survival in the *G. mellonella* infection model

The protective effect of t-CA and FOS was further evaluated in a *G. mellonella* model of UTI89 infection as presented on schematic pipeline (Figure 9A). Infection (5×10^6 cfu/larvae) followed by PBS treatment resulted in progressive larval mortality, whereas untreated non-infected controls remained fully viable throughout the experiment (Figure 9B). FOS monotherapy improved survival in a dose-dependent manner, although the effect remained partial. In contrast, t-CA alone did not significantly improve survival under the tested conditions. The strongest protective effect was observed for the combined treatment. All tested t-CA/FOS combinations maintained survival at, or close to, 100% during the observation period, with the lowest-dose combination showing only a limited decline at the final time point. These results indicate that t-CA markedly enhances the *in vivo* efficacy of FOS against virulent UPEC pathogen UTI89 infection, even though presents limited protective activity when administered alone.

4. Discussion

Recurrent and difficult-to-treat UTIs are strongly associated with UPEC biofilm formation, particularly in hypervirulent strains, where biofilms promote persistence, antimicrobial tolerance, and relapse (Flores-Mireles *et al.*, 2015). This problem is especially relevant in complicated settings, including diabetes and urinary tract instrumentation. Accordingly, FOS remains an attractive therapeutic option because of its broad activity against uropathogens, high

urinary exposure, low side-effect for patients and clinical usefulness in complicated cases. Recent data from Ye et al. showed that fosfomycin trometamol reduced biofilm-forming bacterial colonization on double-J stents in diabetic patients compared with levofloxacin (Ye *et al.*, 2026).

Our results show that t-CA potentiates the antibiofilm activity of FOS. While FOS reduced biofilm growth and viability, t-CA additionally depleted ECM and disrupted biofilm architecture, consistent with our recent transcriptomic data showing downregulation of genes involved in fimbriae and ECM production (Karczewska *et al.*, 2026). This simple mechanistic outcome is relevant because biofilm penetration is a major determinant of antimicrobial efficacy. Dzib-Baak et al. demonstrated that FOS activity against UPEC biofilms depends on the biofilm-producing phenotype, with stronger biofilm producers requiring higher FOS concentrations for biofilm degradation (Dzib-Baak *et al.*, 2022). Thus, t-CA-mediated weakening of the biofilm matrix may improve FOS efficacy by reducing the structural barrier protecting biofilm-associated cells. Conversely, inadequate exposure to FOS may favor biofilm persistence or even enhance biofilm formation under sub-inhibitory conditions, as shown for *Staphylococcus aureus* through a SarA-dependent mechanism (Zeng *et al.*, 2025). This further supports the rationale for combining FOS with an agent that simultaneously reduces biofilm matrix integrity.

The envelope-associated effects observed in our study are consistent with this interpretation. The t-CA–FOS combination affected cell-surface hydrophobicity and membrane potential, but membrane depolarization occurred more slowly than in the colistin-treated control. This suggests that the combination does not act primarily as a rapidly membrane-disrupting, detergent-like treatment. Instead, the slower kinetics may reflect t-CA-driven remodeling of surface-associated structures, local loosening of the cell envelope, and gradual leakage of membrane-potential-sensitive dye. Such an effect would be compatible with reduced ECM abundance, altered biofilm cohesion, and increased physiological stress in biofilm-associated cells.

Together, our data support a model in which t-CA perturbs the pyruvate node rather than acting solely as a membrane-active compound. Docking placed t-CA in the TPP-associated cavity of AceE, close to the catalytic region, while metabolite analysis showed reduced intracellular pyruvate after t-CA treatment. One possible explanation for this apparently counterintuitive decrease is compensatory rerouting of pyruvate through PoxB, whose expression is increased upon t-CA exposure (Chang *et al.*, 1994; Karczewska *et al.*, 2024, 2026). In such a model, PoxB-dependent conversion of pyruvate to acetate and CO₂ could partially bypass the canonical PDH-dependent route to acetyl-CoA and lower the steady-state pyruvate pool (Ogasawara et al., 2007; Sauer & Eikmanns, 2005). Such rerouting may lower the steady-state pyruvate pool, increase acetate-associated acid stress, and engage lysine decarboxylase-dependent pH homeostasis (Moreau, 2007). This interpretation is further supported by recent work in *Streptococcus mutans*, where t-CA was shown to interfere with carbohydrate metabolism, glycolysis, pyruvate metabolism, and the TCA cycle (Zhang *et al.*, 2024). In that study, proteomic analysis, metabolic assays, qRT-PCR, and molecular docking collectively identified PDH as a potential target of t-CA, and t-CA exposure reduced sugar utilization, LDH activity,

and ATP production. The involvement of pyruvate sensing is further supported by our observation that both t-CA and exogenous pyruvate abolished Congo red binding only in the BtsSR-proficient background (Vilhena *et al.*, 2018). Because FOS activity depends on bacterial physiology and drug uptake, including outer-membrane porins and metabolic state, this t-CA-driven metabolic imbalance may reduce the capacity of *E. coli* to tolerate MurA inhibition and thereby potentiate FOS activity (Bianchi *et al.*, 2024). This interpretation is consistent with recent work by Verhulsdonk *et al.*, who showed that the metabolic state of *E. coli* modulates FOS efficacy and promotes resistance evolution. Importantly, their study identified *btsT*, encoding the pyruvate transporter controlled by the BtsSR system, among the upregulated genes associated with post-FOS regrowth (Verhulsdonk *et al.*, 2026). In this context, our observation that both t-CA and exogenous pyruvate abolish Congo red binding in a BtsSR-dependent manner suggests that t-CA intersects with the same pyruvate-sensing axis that can influence recovery after FOS exposure. Thus, t-CA-driven perturbation of pyruvate homeostasis may not only potentiate the immediate antibacterial effect of FOS, but also interfere with metabolic states permissive for regrowth and resistance evolution. This conclusion is further supported by the response of FOS-resistant isolates with different resistance backgrounds (Mueller *et al.*, 2019; Nordmann *et al.*, 2019). Strains carrying genes encoding FosA-type metalloenzymes, including FosA3 and FosA4, did not show detectable re-sensitization to FOS in the presence of t-CA. In contrast, isolate N-279, whose FOS resistance was not associated with enzymatic drug inactivation, displayed restored susceptibility under combined treatment. This distinction suggests that t-CA does not overcome resistance mediated by direct FosA-dependent inactivation of FOS. Rather, its potentiating effect appears to be most relevant when reduced FOS susceptibility is linked to metabolic or physiological adaptation, consistent with the proposed role of t-CA in perturbing pyruvate homeostasis and FOS-responsive recovery pathways.

The present data also suggest that t-CA does not promote FOS resistance under the tested conditions. Although t-CA was previously shown to induce RelA-dependent (p)ppGpp accumulation, this response was not accompanied by activation of major resistance-associated genes, including *marA*, *acrD*, *ampC*, or efflux-related determinants (Karczewska *et al.*, 2024, 2026). It is intriguing in the light of data showing increased antibiotic tolerance upon induction of the stringent response (Spira & Ospino, 2020), and this phenomenon was reported for ciprofloxacin, chloramphenicol, rifampicin, mecillinam, ampicillin, and trimethoprim. For FOS, repeated exposure to t-CA alone did not increase MIC, whereas the combination of t-CA with FOS limited the progressive MIC elevation observed during exposure to FOS alone. Thus, t-CA-induced stress signalling does not appear to translate into a pro-resistance phenotype. This interpretation is consistent with previous studies showing that t-CA can enhance antibacterial activity without promoting stable resistance, including activity against biofilms and multidrug-resistant bacteria (Qiu *et al.*, 2026; Usai & Di Sotto, 2023). Moreover, t-CA was recently shown to target the staphylococcal accessory regulator SarA, thereby enhancing β -lactam activity against methicillin-resistant *Staphylococcus aureus* (Li *et al.*, 2024). Prolonged exposure of t-CA, resulting in parallelly elevated (p)ppGpp level would be considered as a stabilizing factor for DNA repair, thus decreasing the level of spontaneous mutations which could potentially lead to antibiotic resistance (Sivapragasam, 2025; Weaver *et al.*, 2023). In our model, the effects

of t-CA on pyruvate homeostasis, biofilm matrix production, and metabolic recovery may similarly constrain adaptive states required for FOS tolerance and regrowth. However, this conclusion remains provisional and requires further validation, including analysis of evolved populations, FOS transporter expression, and (p)ppGpp dynamics during combined treatment.

The potential clinical relevance of this interaction is supported by the use of two complementary infection-associated models. First, the catheter biofilm model showed that the t-CA/FOS combination was effective against surface-associated UPEC communities, which are directly relevant to catheter-associated and device-associated UTIs. Second, the improved survival of infected *G. mellonella* larvae indicates that the enhanced activity of the combination is not limited to in vitro biofilm assays but is also observed in a living host model. This is relevant because *G. mellonella* is an established surrogate infection model widely used for screening bacterial virulence and evaluating novel antimicrobial strategies, including in UTI-related studies (Chitolina *et al.*, 2025; Karczewska *et al.*, 2023; Pereira *et al.*, 2020). Nevertheless, an important limitation of this study is that the insect model does not reproduce the anatomical, immunological, and pharmacokinetic complexity of mammalian UTIs. However, even if the direct translation of the results obtained in the *G. mellonella* is not possible, also because in this simplified infection model biofilm formation is not usually a key player, it is apparent that the FOS/t-CA combination effect involves decreased virulence even before pathogens can form more complex structure. Therefore, the improved survival of infected larvae should be interpreted as proof-of-concept evidence supporting the in vivo relevance of the t-CA/FOS interaction, rather than as direct evidence of therapeutic efficacy in the urinary tract. Further validation in mammalian UTI models will be required to determine whether t-CA can enhance the therapeutic potential of FOS against biofilm-associated UPEC infections.

5. Conclusions

This study demonstrates that t-CA acts as a phytochemical adjuvant that enhances FOS activity against biofilm-forming *E. coli*, including UPEC strains. The main novelty of this work is the identification of a dual mode of potentiation: t-CA weakens the biofilm matrix while simultaneously perturbing bacterial metabolic adaptation at the pyruvate node. This combined antibiofilm and metabolic effect increased FOS efficacy, improved the outcome in an in vivo infection model, and, importantly, limited the emergence of reduced FOS susceptibility during repeated exposure. Our findings support a model in which plant-derived metabolites can enhance antibiotic activity not only by direct antibacterial or antibiofilm effects, but also by restricting bacterial physiological plasticity and adaptive recovery. By targeting both biofilm-associated protection and metabolic states permissive for FOS tolerance, t-CA may help reduce the resistance-developing potential of FOS-based treatment strategies. Nonetheless, further validation in mammalian UTI models and evolved bacterial populations will be required to confirm the therapeutic relevance and resistance-limiting potential of this approach.

6. Acknowledgments

We are grateful to prof. Kirsten Jung for providing *btsSR* and *btsT* *E. coli* mutants used in this work, prof. Patrice Nordmann for providing fosfomycin resistant UPEC isolates and Dorota Łuszczek for her technical support with SEM imaging.

7. Authors Contribution

Monika Karczewska: Conceptualization, Investigation, Methodology, Data Curation, Visualization, Writing – Original Draft. **Patryk Strzelecki:** Investigation. **Monika Maciąg-Dorszyńska:** Investigation, Methodology, Data Curation. **Malgorzata Kapusta:** Investigation, Methodology, Data Curation. **Agnieszka Pyrczak-Felczykowska:** Investigation, Methodology. **Agnieszka Szalewska-Palasz:** Methodology, Validation, Supervision, Writing – Review & Editing. **Dariusz Nowicki:** Conceptualization, Investigation, Methodology, Validation, Visualization, Funding Acquisition, Supervision, Writing – Original Draft, Writing – Review & Editing.

8. Declaration statement

We declare no conflicts of interest related to this study.

9. Data availability

All original data generated during the study are included in the article in the form of figures and tables. For any further inquiries, please contact the corresponding author.

10. Funding

This work was supported by the National Science Centre SONATA grant no. UMO-2018/31/D/NZ7/02258 for DN.

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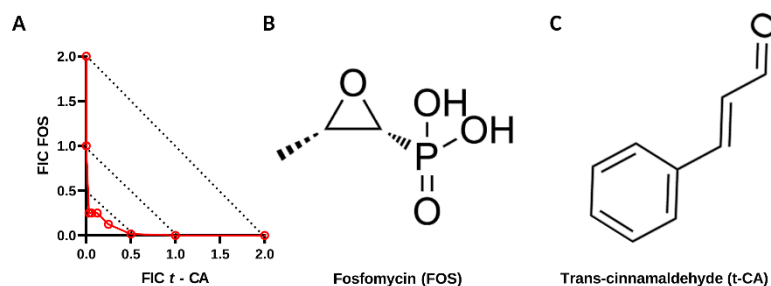
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842 Figures and descriptions

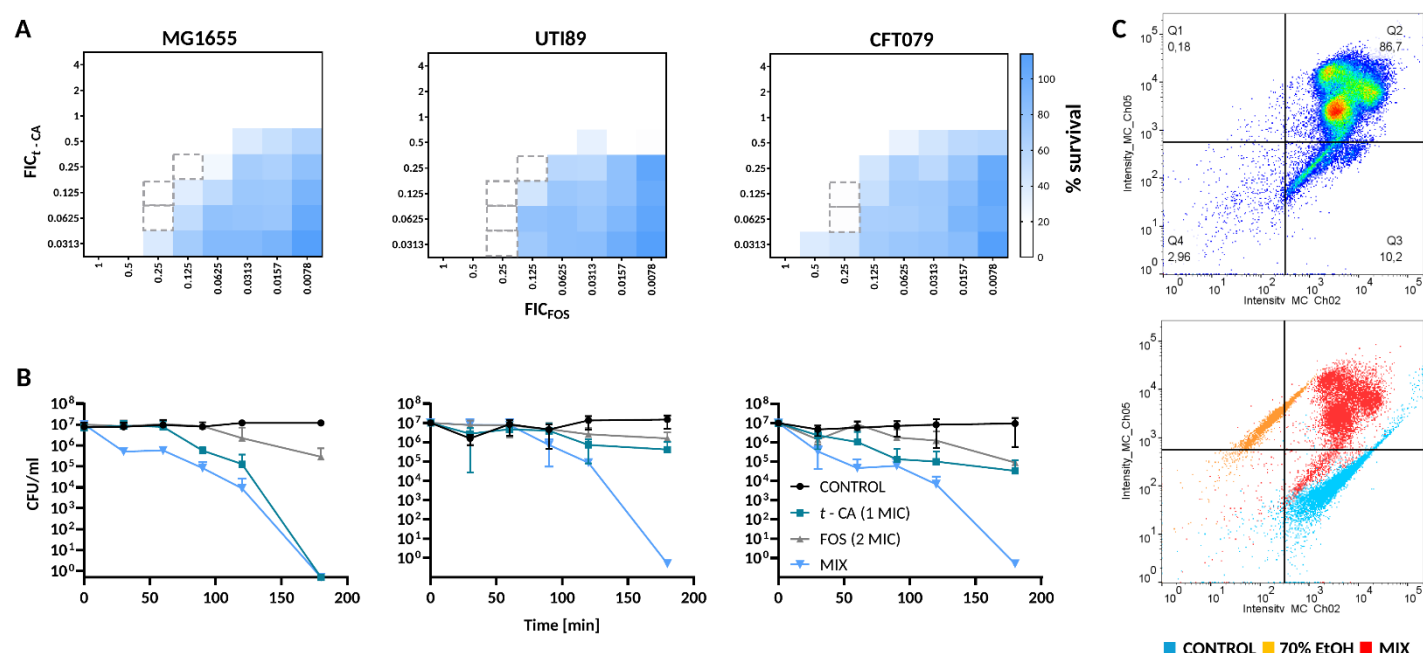


843

844 **Figure 1. Fosfomycin/trans-cinnamaldehyde interaction and chemical structures of the**
 845 **tested compounds.** (A) Representative isobologram of the interaction between fosfomycin
 846 (FOS) and trans-cinnamaldehyde (t-CA) against *E. coli* MG1655. Fractional inhibitory
 847 concentration (FIC) values are shown for FOS and t-CA, and dotted diagonal lines indicate the
 848 thresholds used for fractional inhibitory concentration index (FICI) classification. The red
 849 interaction curve falls within the synergistic range, indicating potentiation of FOS activity by t-
 850 CA. (B) Chemical structure of FOS, a MurA-targeting antibiotic that inhibits peptidoglycan
 851 biosynthesis. (C) Chemical structure of t-CA, a plant-derived phenylpropanoid evaluated as a
 852 phytochemical adjuvant.

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Figure 2. Trans-cinnamaldehyde potentiates the antibacterial activity of fosfomycin against *Escherichia coli*. (A) Checkerboard survival maps showing the interaction between fosfomycin (FOS) and trans-cinnamaldehyde (t-CA) against *E. coli* MG1655 and the uropathogenic *E. coli* (UPEC) strains UTI89 and CFT073. The x-axis represents the fractional inhibitory concentration (FIC) of FOS, whereas the y-axis represents the FIC of t-CA. Color intensity indicates bacterial survival, and dashed rectangles mark concentration combinations corresponding to synergistic interactions. (B) Time-kill kinetics of MG1655, UTI89, and CFT073 exposed to t-CA, FOS, or their combination. Bacterial survival was monitored over 180 min and expressed as colony-forming units per milliliter (CFU/mL). (C) Flow cytometric analysis of membrane integrity after treatment (120 min) with the FOS/t-CA combination (0.5 x 0.5 MIC). UTI89 cells were stained with SYTO 9 and propidium iodide (PI). Untreated live cells and ethanol-killed cells were used as controls. The combination (MIX) shifted the bacterial population toward the PI-positive fraction, indicating extensive membrane damage and loss of viability.

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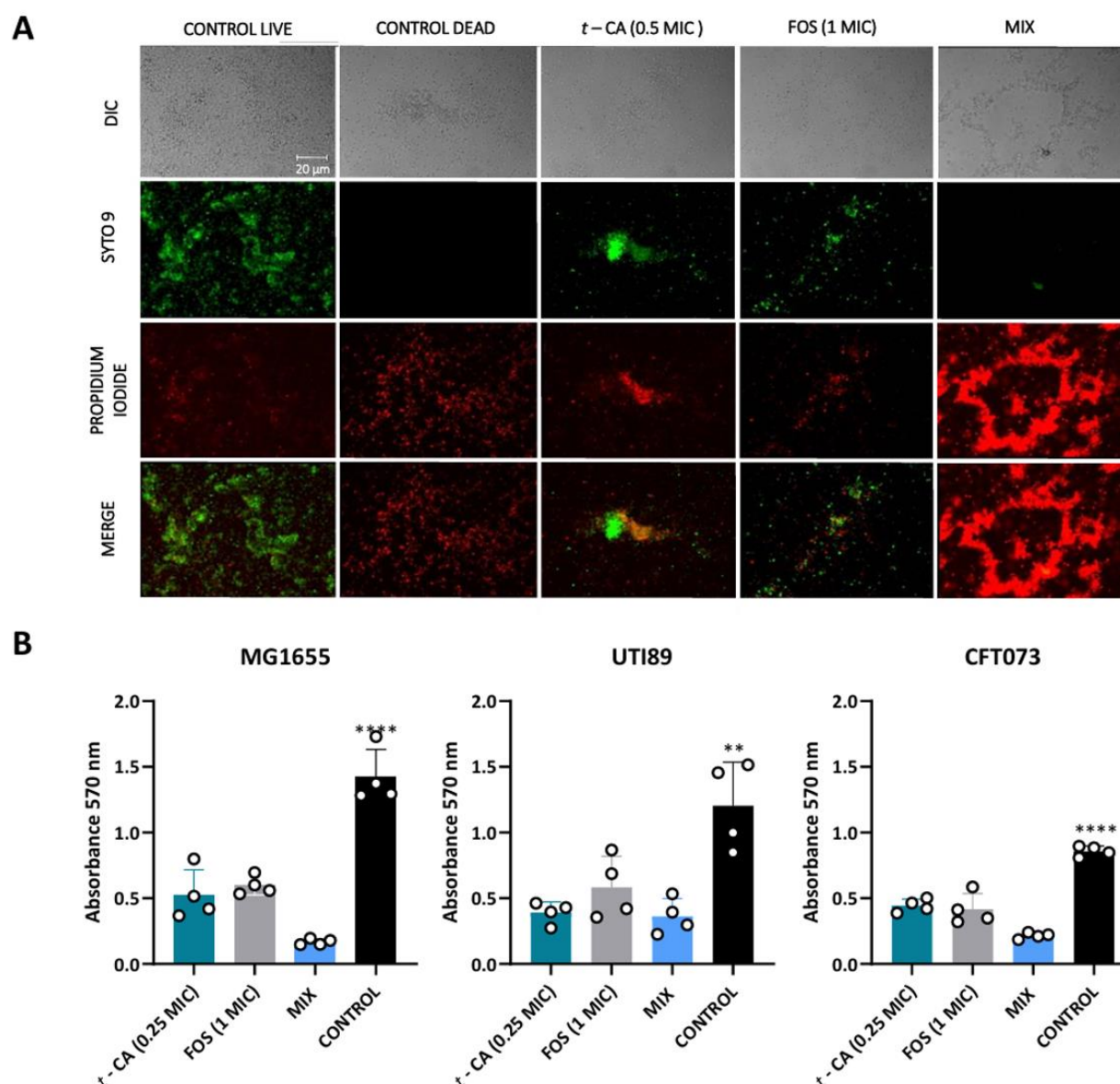
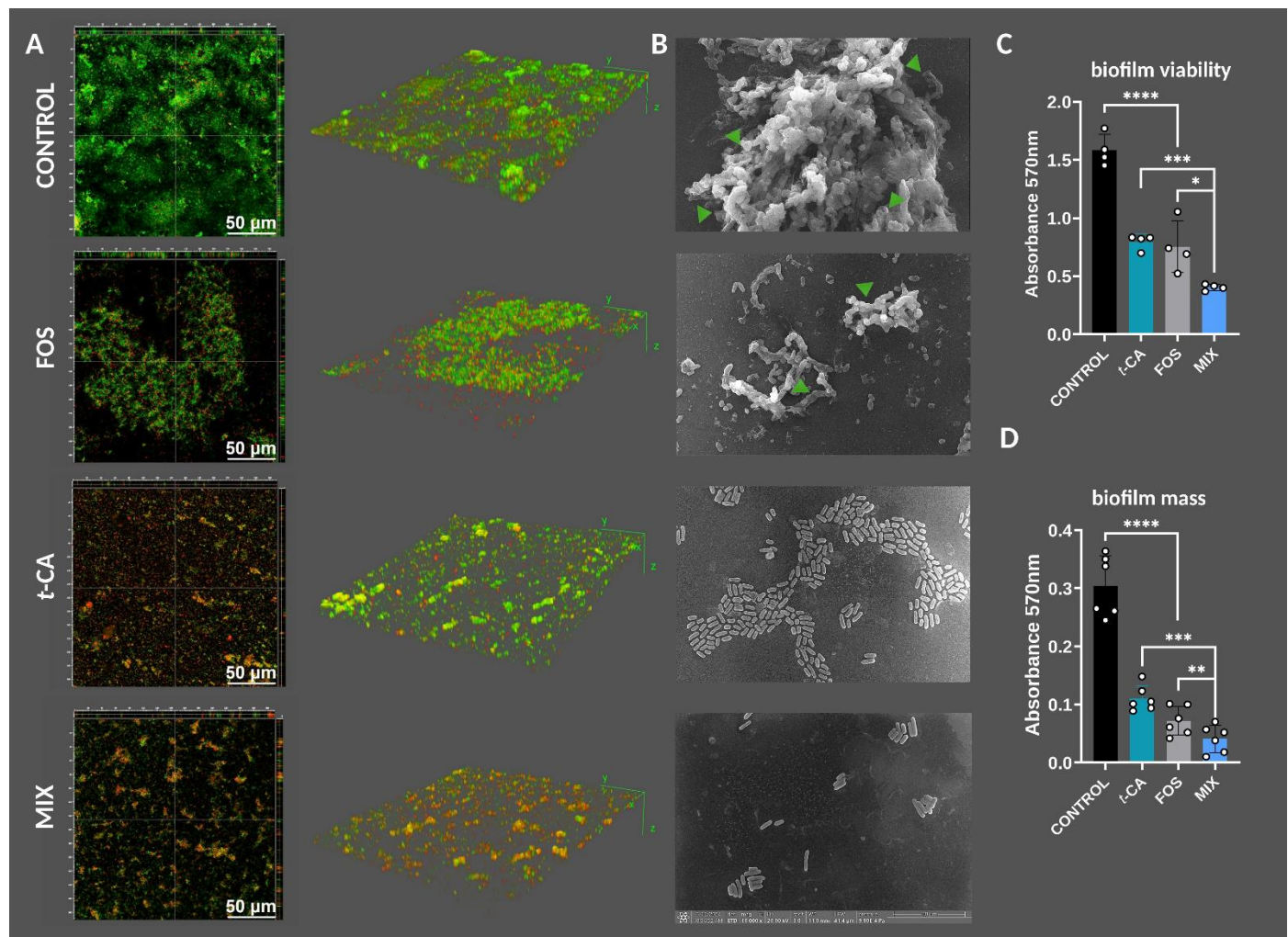


Figure 3. Trans-cinnamaldehyde enhances the antibiofilm activity of fosfomycin in a 96-well plate biofilm model. (A) Representative fluorescence microscopy images of UTI89 biofilms stained with SYTO 9 and propidium iodide (PI) after treatment with trans-cinnamaldehyde (t-CA; 0.5×MIC), fosfomycin (FOS; 1×MIC), or their combination (MIX). Untreated live biofilms and ethanol-killed biofilms were included as staining controls. DIC, SYTO 9, PI, and merged channels are shown. SYTO 9-positive cells are shown in green, whereas PI-positive cells are shown in red and indicate membrane-compromised or dead cells. Scale bar: 20 μ m. (B) Metabolic activity of biofilms formed by *Escherichia coli* MG1655 and the uropathogenic *E. coli* strains UTI89 and CFT073, determined using the MTT reduction assay. Biofilms were exposed to t-CA, FOS, or MIX, and absorbance was measured at 570 nm. Data are presented as mean $\pm$ SD from independent biological replicates. Statistical significance was assessed relative to the untreated control; **p < 0.01, ***p < 0.001, ****p < 0.0001.



885

886 **Figure 4. The trans-cinnamaldehyde/fosfomycin combination disrupts three-dimensional**
887 **biofilm architecture and catheter-associated UPEC biofilms.** (A) Representative confocal
888 laser scanning microscopy images and three-dimensional reconstructions of UTI89 biofilms
889 treated with fosfomycin (FOS), trans-cinnamaldehyde (t-CA), or their combination (MIX).
890 Biofilms were stained with SYTO 9 and propidium iodide (PI). SYTO 9-positive cells are
891 shown in green, whereas PI-positive membrane-compromised or dead cells are shown in red.
892 Scale bar: 50 μ m. (B) Representative scanning electron microscopy images of catheter-
893 associated UTI89 biofilms after treatment with FOS, t-CA, or MIX. Green arrowheads indicate
894 bacterial aggregates and extracellular biofilm material. (C) Biofilm viability determined by the
895 MTT reduction assay and expressed as absorbance at 570 nm. (D) Total biofilm biomass
896 quantified by crystal violet staining and expressed as absorbance at 570 nm. Data are presented
897 as mean $\pm$ SD from independent biological replicates. Statistical significance was assessed as
898 indicated in the figure; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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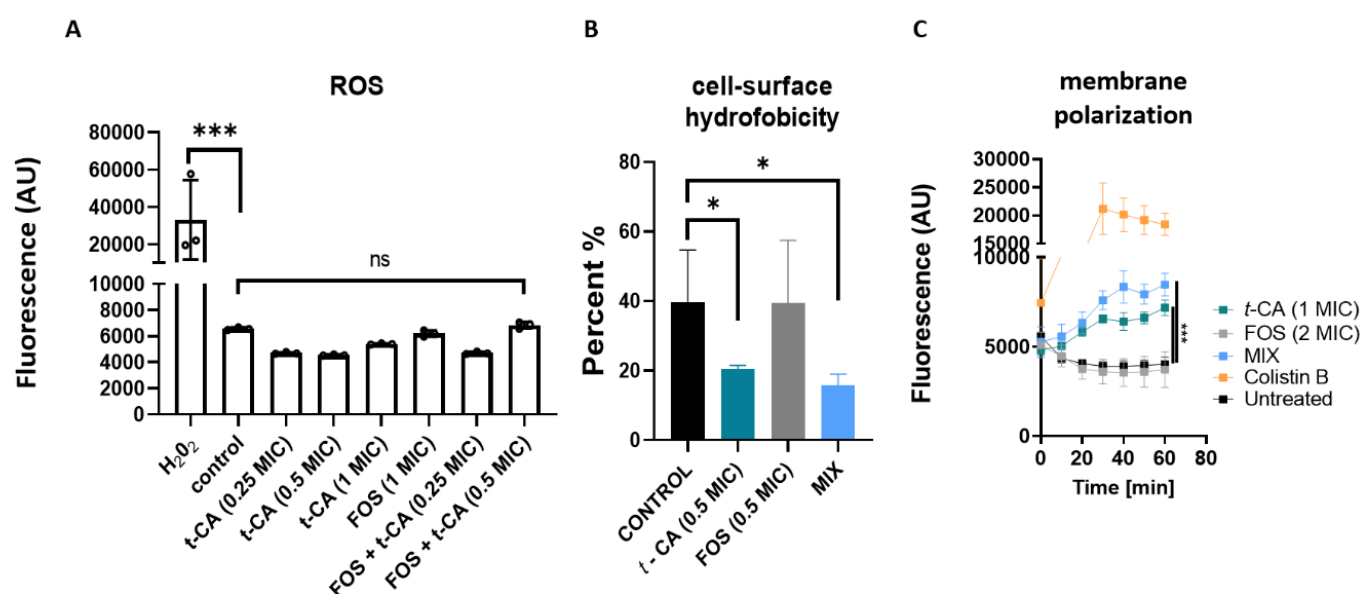
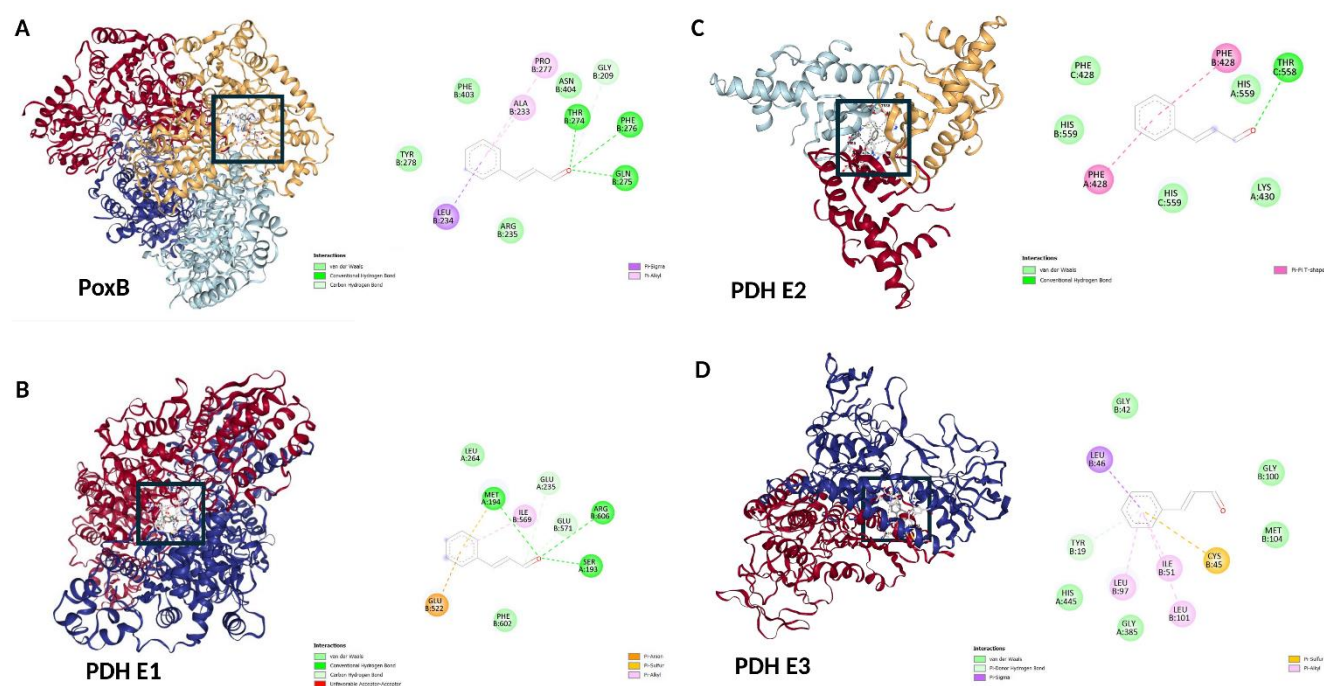


Figure 5. Trans-cinnamaldehyde alters cell-surface properties and membrane polarization without inducing oxidative stress. (A) Intracellular reactive oxygen species (ROS) production in UTI89 cells exposed to trans-cinnamaldehyde (t-CA), fosfomycin (FOS), or their combination, measured using 2',7'-dichlorofluorescein diacetate (DCFDA). Hydrogen peroxide (H₂O₂) was used as a positive control. (B) Cell-surface hydrophobicity determined using the microbial adhesion to hydrocarbons (MATH) assay. Results were determined spectrophotometrically (A₅₅₀) and presented as a percent of hydrophobicity, tested against untreated control (C) Changes in membrane polarization monitored using the membrane-potential-sensitive fluorescent probe DiSC₃(5). Colistin B was used as a positive control of depolarization. Data are presented as mean ± SD from independent biological replicates. Statistical significance is indicated in the figure; ns, not significant; *p < 0.05, ***p < 0.001.

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Figure 6. Predicted interactions of trans-cinnamaldehyde with enzymes operating at the pyruvate node. Molecular docking analysis of trans-cinnamaldehyde (t-CA) with enzymes involved in pyruvate metabolism in *E. coli*. Predicted binding poses are shown for (A) pyruvate oxidase (PoxB), (B) the E1 component of the pyruvate dehydrogenase complex (PDH E1; AceE), (C) the E2 component of the pyruvate dehydrogenase complex (PDH E2; AceF), and (D) the E3 component of the pyruvate dehydrogenase complex (PDH E3; LpdA).

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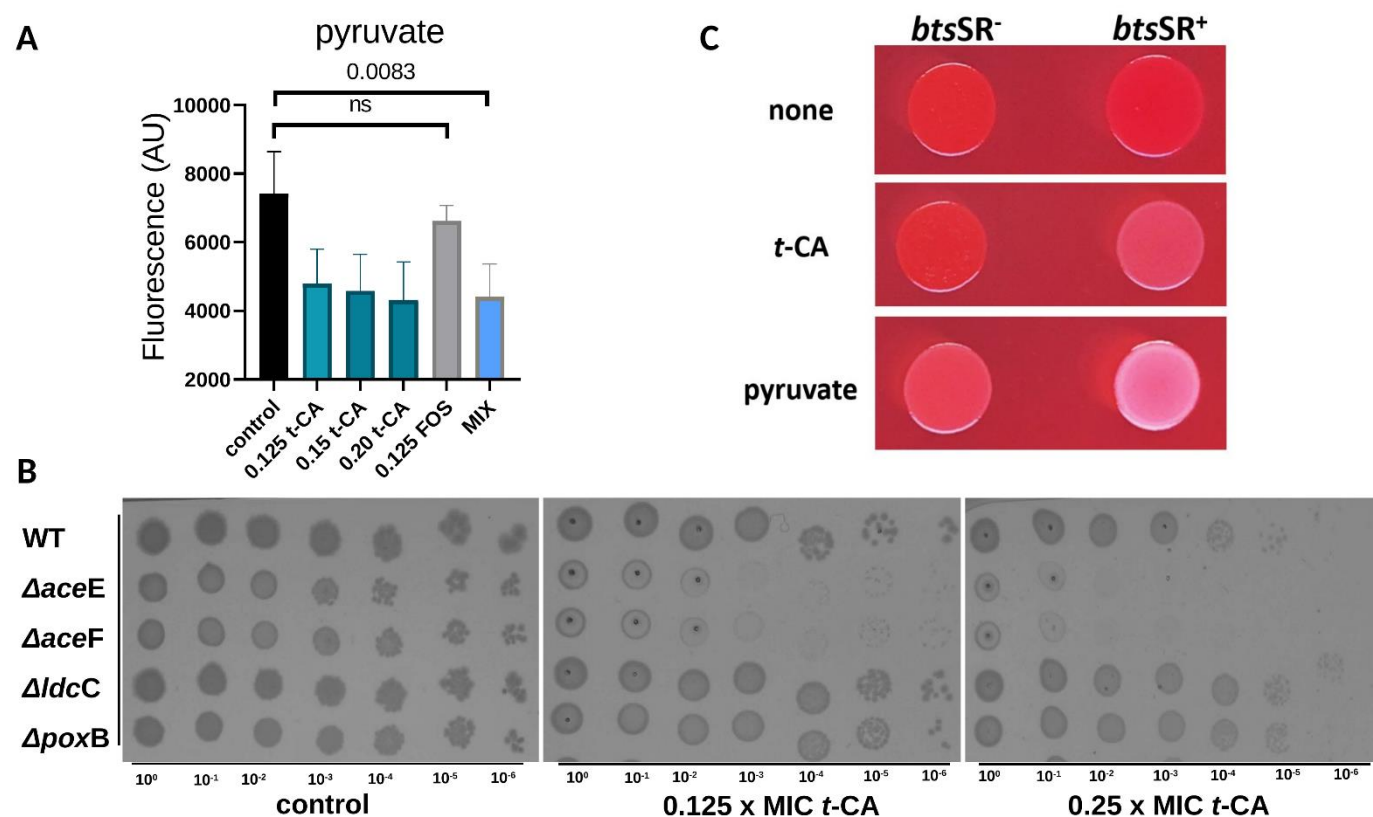


Figure 7. Trans-cinnamaldehyde perturbs pyruvate homeostasis and reveals the contribution of pyruvate metabolism and sensing to the bacterial response. (A) Intracellular pyruvate levels in *E. coli* cells exposed to subinhibitory concentrations of trans-cinnamaldehyde (t-CA), fosfomycin (FOS), or their combination (MIX). Pyruvate levels were measured fluorometrically (Ex:530, Em:585) and expressed as arbitrary fluorescence units (AU). Statistical significance is indicated in the figure; ns, not significant. (B) Serial dilution spot assay of *E. coli* MG1655 wild type (WT) and deletion mutants lacking selected metabolic functions: $\Delta aceE$, $\Delta aceF$, $\Delta ldcC$, and $\Delta poxB$. Fresh cultures ($OD_{600} = 0.5$) were serially diluted from 10^0 to 10^{-6} and spotted onto control plates or plates containing t-CA at 0.125×MIC or 0.25×MIC. (C) Congo red binding phenotype of strains differing in the BtsSR pyruvate-sensing system. Colony phenotypes of *btsSR*⁻ and *btsSR*⁺ backgrounds are shown under untreated conditions, after exposure to t-CA (0.125×MIC), or after supplementation with exogenous pyruvate (60μM). Loss of Congo red binding in the *btsSR*⁺ background indicates that the effect of t-CA and pyruvate on matrix-associated colony phenotype depends on the BtsSR pyruvate-sensing system.

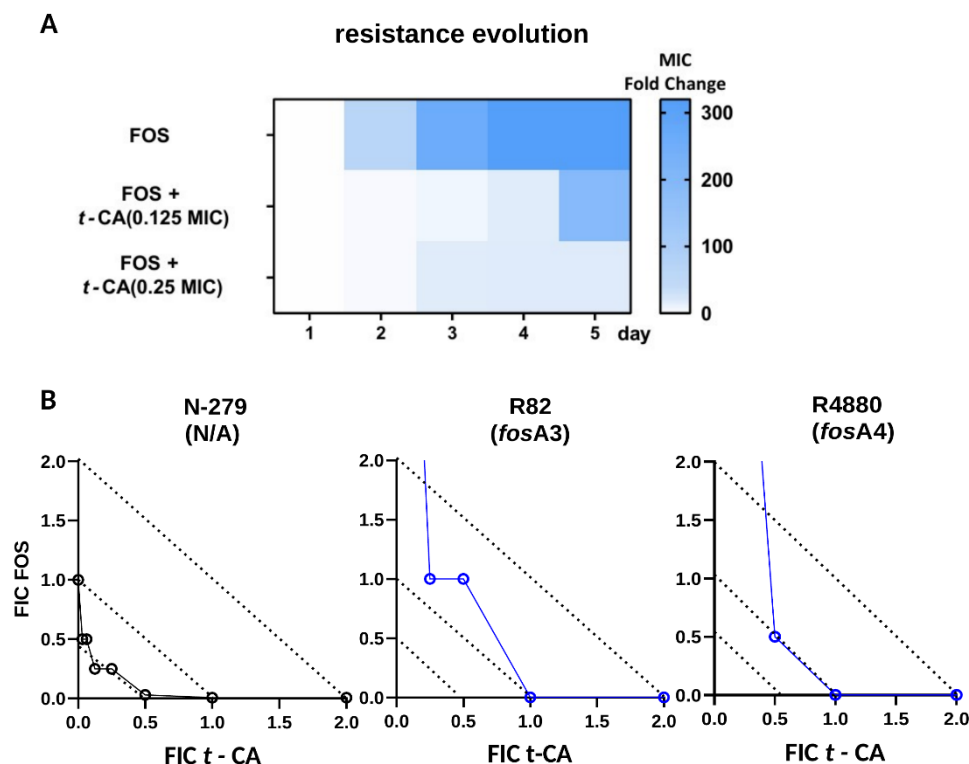


Figure 8. Trans-cinnamaldehyde limits the development of reduced fosfomycin susceptibility and potentiates fosfomycin activity in isolates without FosA-mediated resistance. (A) Experimental evolution of fosfomycin (FOS) susceptibility during serial exposure. *E. coli* cultures were passaged for 5 consecutive days in the presence of FOS alone or FOS combined with trans-cinnamaldehyde (t-CA) at 0.125×MIC or 0.25×MIC. (B) Isobolograms show the interaction between FOS and t-CA in FOS-resistant clinical isolates with different resistance backgrounds (N-279, which lacked detectable *fosA1–fosA6* determinants, R82 and R4880, carrying the FosA-type metalloenzyme genes *fosA3* and *fosA4*, respectively).

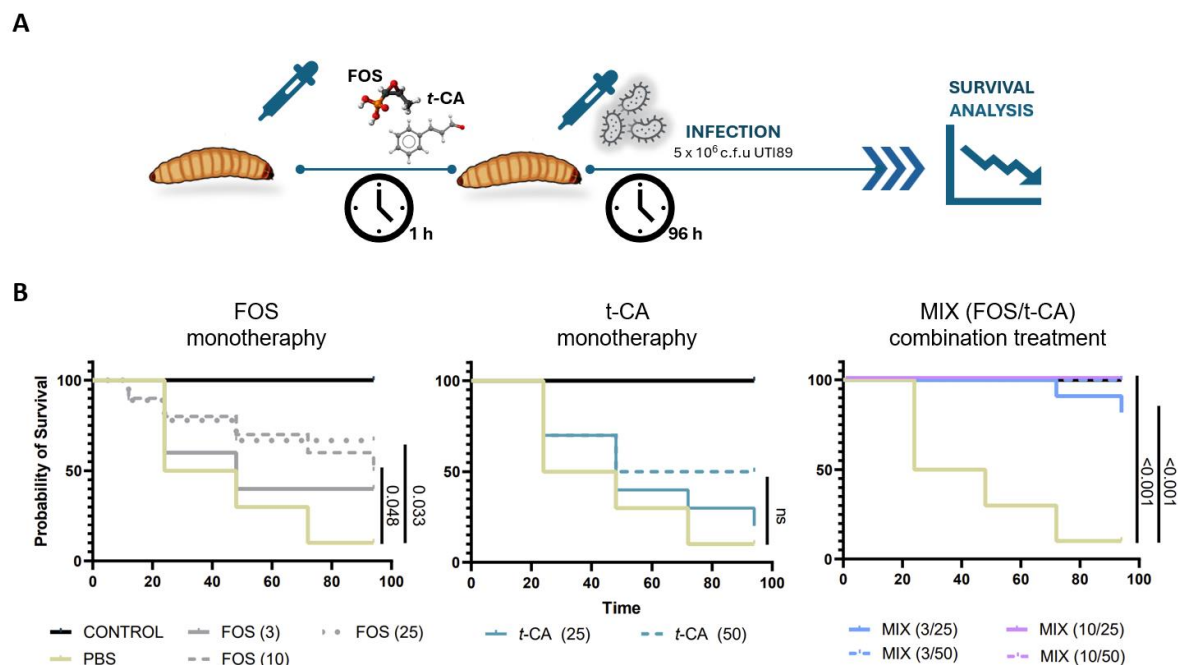


Figure 9. The trans-cinnamaldehyde/fosfomycin combination improves survival in a *Galleria mellonella* model of UTI89 infection. (A) Schematic representation of the experimental workflow. *G. mellonella* larvae were pre-treated with fosfomycin (FOS), trans-cinnamaldehyde (t-CA), or their combination. After 1 h, larvae were infected with the uropathogenic *E. coli* strain UTI89 at 5×10^6 colony-forming units (CFU) per larva. Survival was monitored for 96 h. (B) Kaplan-Meier survival curves of infected larvae treated with FOS, t-CA, or the FOS/t-CA combination. PBS-treated infected larvae and non-infected untreated larvae were included as controls. All doses used in experiment are expressed as mg/kg body weight. Statistical significance was assessed using the log-rank test; ns, not significant.



Review

Phytochemicals Controlling Enterohemorrhagic *Escherichia coli* (EHEC) Virulence—Current Knowledge of Their Mechanisms of Action

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Abstract: Enterohemorrhagic *Escherichia coli* (EHEC) is a common pathotype of *E. coli* that causes numerous outbreaks of foodborne illnesses. EHEC is a zoonotic pathogen that is transmitted from animals to humans. Ruminants, particularly cattle, are considered important reservoirs for virulent EHEC strains. Humans can become infected with EHEC through the consumption of contaminated food and water or through direct contact with infected animals or humans. *E. coli* O157:H7 is one of the most commonly reported causes of foodborne illnesses in developed countries. The formation of attaching and effacing (A/E) lesions on the intestinal epithelium, combined with Shiga toxin production, is a hallmark of EHEC infection and can lead to lethal hemolytic–uremic syndrome (HUS). For the phage-dependent regulation of Shiga toxin production, antibiotic treatment is contraindicated, as it may exacerbate toxin production, limiting therapeutic options to supportive care. In response to this challenge and the growing threat of antibiotic resistance, phytochemicals have emerged as promising antivirulence agents. These plant-derived compounds target bacterial virulence mechanisms without promoting resistance. Therefore, the aim of this study is to summarize the recent knowledge on the use of phytochemicals targeting EHEC. We focused on the molecular basis of their action, targeting the principal virulence determinants of EHEC.

Keywords: Shiga toxin; foodborne pathogens; *E. coli* O157:H7; bacteriophages; antimicrobial therapy; isothiocyanates; cinnamaldehyde; terpenes; polyphenols; biofilm



Academic Editors: Simon Vlad Luca and Adriana Trifan

Received: 20 November 2024

Revised: 29 December 2024

Accepted: 2 January 2025

Published: 4 January 2025

Citation: Strzelecki, P.; Karczewska, M.; Szalewska-Pałasz, A.; Nowicki, D.

Phytochemicals Controlling

Enterohemorrhagic *Escherichia coli*

(EHEC) Virulence—Current

Knowledge of Their Mechanisms of

Action. *Int. J. Mol. Sci.* **2025**, *26*, 381.

[https://doi.org/10.3390/](https://doi.org/10.3390/ijms26010381)

[ijms26010381](https://doi.org/10.3390/ijms26010381)

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1. Introduction

Most known *Escherichia coli* strains are a part of the natural microbiome of human intestinal flora. Pathogenic *E. coli* strains are broadly classified based on their virulence factors and disease characteristics. The two major distinctions of pathogenic *E. coli* strains are non-diarrheagenic and diarrheagenic [1]. Pathogenic *E. coli* strains unrelated to gastroenteritis are mainly referred to as extraintestinal pathogenic *E. coli* (ExPEC), which are the etiological agent of infection outside the host's gastrointestinal tract. ExPEC members comprise, among others, uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC), and neonatal meningitis-associated *E. coli* (NMEC) [1]. Diarrheagenic *E. coli* strains are associated with severe gastroenteritis and include the Shiga toxin-producing *E. coli* (STEC) and enterohemorrhagic *E. coli* (EHEC) groups that are the focus of this review. *E. coli* O157:H7, the most prominent EHEC member, has an extremely low infectious dose, estimated

at 10 to 100 cells. Infections typically affect the elderly and children due to insufficiency of the immune system. However, the German outbreak in 2011 showed that age is not a limiting factor in the development of EHEC infections, as many of the several cases involved young and previously healthy persons [2]. Gastrointestinal symptoms usually manifest within 3–4 days post infection. The most common complication is hemorrhagic colitis, which is characterized by abdominal cramps and bloody diarrhea. STEC strains are estimated to cause more than 265,000 cases annually only in the United States, making them among the world's greatest microbial threats. More serious sequela of EHEC infection is hemolytic-uremic syndrome (HUS), leading to hemolytic anemia, acute renal failure and thrombocytopenia, and subsequent kidney disfunction, resulting in the patient's death or the necessity of kidney transplants [3].

The EHEC virulence factor that distinguishes HUS-inducing strains from other *E. coli* pathovars is Shiga toxin production. There are two genetically distinct types of Shiga toxins, Stx1 and Stx2, each with several subtypes. Stx2a and Stx2c are commonly associated with HUS development in humans. Shiga toxin belongs to the AB₅ toxin family, which binds specific glycosphingolipids on human cells, including glomerular and brain endothelial cells. These receptors include globotriaosylceramide (Gb3, CD77), and to a lesser extent, globotetraosylceramide (Gb4). Stx toxins cause cell death by inhibiting protein translation [4]. Manifestations of systemic intoxication include vascular dysfunction and thrombus formation, leading to HUS. Furthermore, the genes encoding Stx are located on the lambdoid prophage, making treatment difficult (Figure 1). Namely, the use of antibiotics, especially those from the quinolone family, can worsen the symptoms of infection by triggering a bacterial SOS response and subsequently excising the phage genetic material along with the transcription of phage-related genes [5]. In this context, virulence factors such as Shiga toxin will be released even if antibiotics effectively inhibit bacterial growth. Therefore, the treatment of Shiga toxigenic infections is currently limited to supportive therapies such as rehydration and dialysis. However, new therapeutic approaches are being developed to improve management of infection and disease by targeting virulence.

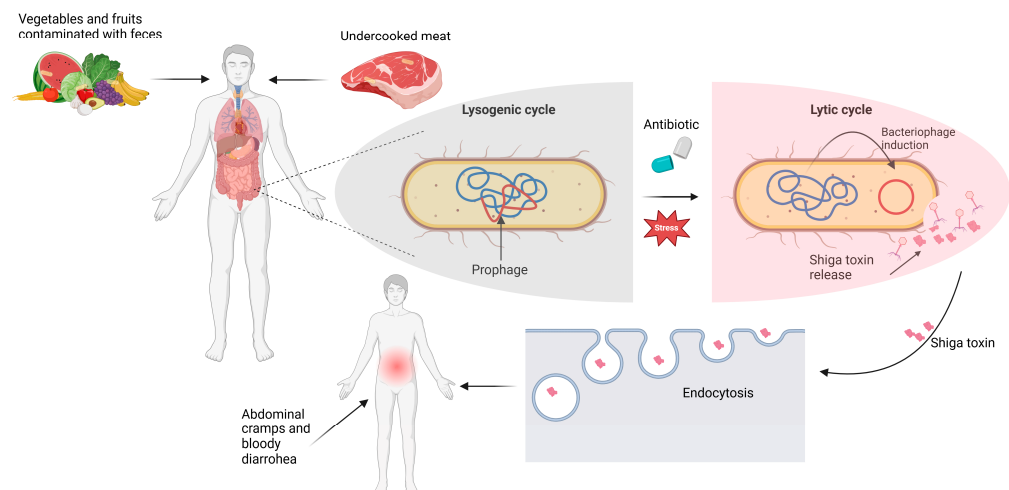


Figure 1. Mechanism of enterohemorrhagic *Escherichia coli* (EHEC) infection and Shiga toxin production. The scheme outlines the infection pathway following the ingestion of contaminated food, such as vegetables, fruits, or undercooked meat. EHEC carries a prophage that remains latent during the lysogenic cycle. Upon exposure to environmental stressors, including antibiotic treatment, the prophage is activated, triggering the lytic cycle and subsequent Shiga toxin production. The toxin enters host cells through endocytosis, leading to severe gastrointestinal symptoms, including abdominal cramps and bloody diarrhea. The presented scheme was prepared based on [4,5]. Created in BioRender. <https://BioRender.com/y08b332>.

Nowadays, a resolute effort should be made to search for new antimicrobial agents that allow us to replace known antibiotics, as they are losing their effectiveness against multidrug-resistant pathogens. Plant-derived secondary metabolites provide a very diverse framework that has been used to design new drugs, including antimicrobials [6]. In nature, secondary metabolites contribute to the systemic and induced defense of plants against bacteria, viruses, and fungi. Several phytochemicals belonging to classes such as coumarins, flavonoids, terpenoids, and alkaloids show inhibitory properties against many microorganisms. However, there is still a gap in the knowledge to be filled concerning the mechanistic principles of their action against specific pathogens. Thus, the aim of this work was to summarize the current studies on the activity of phytochemicals against Shiga toxinogenic *E. coli*.

2. EHEC Virulence Factors

2.1. Shiga Toxin Production

The genes responsible for encoding Shiga toxins (*stx* genes) do not originate from the bacterial chromosome. Instead, these genes are incorporated into the genetic material of lambdoid bacteriophages (viruses related to the λ family) that have the ability to lysogenize *E. coli* strains. These particular phages are referred to as Shiga toxin-converting bacteriophages [7]. The expression of *stx* genes is directly dependent on the phage lytic cycle, which in turn is highly sensitive to environmental stimuli. Generally, the expression of nearly all phage genes, except for the *cl* gene, is significantly suppressed when the phage exists in the prophage state, due to the action of the CI repressor [5,8]. This repressor inhibits the major early phage promoters, pL and pR, which are essential for the expression of genes involved in the lytic cycle. One such gene, Q, encodes an anti-termination protein necessary for efficient transcription from the pR' promoter, responsible for expression of so-called late-phage genes [8]. Transcripts originating from the pR' promoter direct the synthesis of proteins involved in host cell lysis, along with structural proteins needed for capsid assembly. In Shiga toxin-converting bacteriophages, the *stx* genes are located between pR' and the lysis genes [7,8]. Therefore, in lysogenized bacteria, the expression of *stx* genes is repressed, and the production of Shiga toxins can only occur following prophage induction [9,10]. Moreover, after prophage induction, the efficient expression of *stx* genes relies on successful phage DNA replication, leading to the amplification of these genes within the host cell [7].

2.2. LEE (Locus of Enterocyte Effacement)

In addition to the production of Stx toxin, the pathogenesis of EHEC is also characterized by the bacterium's ability to colonize the human intestine [11,12]. After entering the gastrointestinal tract, EHEC migrates to the distal ileum and colon, where it colonizes these organs by forming strong attachments to the surface of epithelial cells. At sites of bacterial attachment to colonocytes, EHEC induces the formation of F-actin-rich pedestals directly beneath the attached bacteria [11]. Genes encoded on a pathogenicity island (PAI) within the locus of enterocyte effacement (LEE) are responsible for this characteristic EHEC phenotype. The LEE region contains 41 open reading frames organized into five polycistronic operons, LEE1 to LEE5. LEE expression is tightly regulated and encodes the type 3 secretion system (T3SS). The T3SS acts like a molecular syringe, delivering bacterial effector proteins directly into the cytoplasm of host cells [13]. The presence of LEE had been demonstrated also for other enteropathogenic *E. coli*, *Citrobacter rodentium*, and *E. albertii* [14].

When EHEC makes close contact with host epithelial cells, it employs the T3SS to inject dozens of effector proteins into the host cell's cytoplasm. One key effector is the translocated intimin receptor (Tir), which integrates into the host plasma membrane and serves as a

receptor for intimin, a bacterial outer membrane protein. The interaction between intimin and Tir facilitates bacterial attachment and triggers actin polymerization, resulting in the formation of characteristic attachment and effacement (A/E) lesions. The expression of LEE is controlled by the master regulator Ler, encoded by the LEE1 operon, and is further modulated by multiple transcription factors in response to environmental cues. Recent reviews have extensively covered the regulation of EHEC virulence factors [13,15,16].

2.3. Biofilm

Although biofilm formation is not a primary virulence feature of EHEC, its multicellular structure helps bacteria survive under unfavorable conditions [17]. The ability of *E. coli* O157:H7 to attach to abiotic and biotic surfaces through specific virulence factors—such as adhesins, flagella, type I fimbriae, curli fibers, and the enterocyte effacement locus—is a complex and critical step in infection initiation and biofilm formation [18]. This process is influenced by various factors, including the hydrophobicity and charge of the cell surface, as well as the amount and distribution of extracellular polymeric substances. Flagella enable bacterial motility and help EHEC overcome repulsive electrostatic forces between surfaces and cells, facilitating adhesion and biofilm formation on both abiotic and biotic surfaces [19]. Curli are thin, coiled fibers of varying lengths that self-assemble outside the bacterial cell and form an amorphous matrix essential for colonization and biofilm development. During attachment, the genes responsible for curli production are upregulated, enhancing surface association and providing protection against unfavorable environmental conditions. The production of curli polymers is environmentally regulated and depends on RpoS (sigma S subunit of RNA polymerase), meaning that *csgA* (the structural subunit of curli) transcription is controlled by environmental factors such as low temperature, osmolarity, and stationary phase growth conditions.

Adhesins, outer membrane proteins (such as intimin), and long polar fimbriae (LPF) play important roles in the colonization of human intestinal epithelial cells [16]. The bacterium has two chromosomal operons encoding LPF, which contribute to adherence in vitro and colonization in vivo. It also forms exopolysaccharides (EPSs) that act as a physical barrier to protect cells from environmental stressors such as temperature changes, nutrient limitations, pH changes, and biocide exposure. EPS is also involved in cell adhesion and biofilm formation, acting as a conditioning layer or adhesive on abiotic surfaces such as stainless steel.

The formation of biofilms by EHEC bacteria is regulated by quorum sensing (QS). QS is a system that globally controls the expression of genes involved in virulence factor secretion and biofilm maturation [20]. Bacterial cells secrete signaling molecules (autoinducers) into their surrounding environment. Once a critical density has been attained, the cells initiate the process of biofilms forming and maturing. The chemotactic attraction of EHEC by AI-2, a key autoinducer, is mediated by two receptors, LsrB and LsrR, which regulate motility, cell attachment, and biofilm formation. *E. coli* can interfere with the AI-2-regulated behavior of other bacterial species in an Lsr-dependent manner. The signaling properties of AI-2 enhance the adaptability of both Gram-positive and Gram-negative bacteria to various environmental conditions, promoting adhesion and biofilm formation, which in turn contributes to pathogenicity. AI-2, known as a “universal autoinducer”, regulates bacterial physiology and impacts virulence factor production and biofilm formation in certain bacteria. Another signaling molecule associated with *E. coli* O157:H7 is autoinducer-3 (AI-3), which requires tyrosine for synthesis and depends on LuxS to activate the transcription of pathogenicity genes located within the LEE.

3. Phytochemicals with Antimicrobial Activity Against EHEC

The treatment of EHEC infections poses unique challenges, as antibiotics can exacerbate disease severity by inducing Shiga toxin production machinery, as was mentioned above. This highlights the need for alternative therapeutic strategies that target bacterial pathogens through mechanisms distinct from existing antibiotics to avoid the risk of worsening a patient's condition due to the treatment [21]. Antivirulence strategies represent an innovative alternative by targeting specific bacterial virulence mechanisms, such as toxin production, adhesion, or biofilm formation, without directly affecting bacterial survival [22–24]. This approach minimizes the risk of selective pressure that drives the emergence of antibiotic resistance and reduces the potential disruption of the host microbiome. Unlike conventional antibiotics, antivirulence agents are designed to disarm the pathogen, thereby mitigating its ability to cause disease while allowing the host immune system to overcome infection. This strategy is particularly critical in the face of the growing ineffectiveness of broad-spectrum antibiotics. Plant-derived phytochemicals, with their diverse structures and bioactive properties, represent a particularly promising class of antivirulence agents, which warrants further exploration of their mechanisms and therapeutic potential not only in the context of EHEC infections. In the following chapters, we focus on describing the anti-virulent properties of selected phytochemicals.

3.1. Isothiocyanates

Isothiocyanates (ITCs) are the group of compounds containing the monovalent anion $[N=C=S]^-$, with the common formula of $R-N=C=S$; chemically, they are salts or esters of isocyanic acid. These secondary plant metabolites are considered stress-response plant chemicals. They are abundant in plants from the *Brassicaceae* family, particularly in the *Brassica* genus. Their precursors, glucosinolates, are hydrolyzed by the enzyme β -thioglucosidase (myrosinase), which is released upon plant tissue injury as a protection against the source of damage [25]. Another source of myrosinase, especially important for dietary ITCs, is the intestine microbiome; therefore, cooked vegetables, devoid of active myrosinase, can still be a source of ITCs. The popularity of *Brassicaceae* plants in the human diet in all regions of culture and climate (e.g., broccoli, cabbage, horseradish, kale, cauliflower, turnip, watercress, mustard, bok choy, and many others) has made ITCs a part of daily phytochemical intake and promoted the widespread knowledge of their health benefits. Each of the various glucosinolates present in plants leads to the formation of a different ITC upon hydrolysis, providing a variety of these bioactive compounds with particular characteristics (Figure 2). The increasing attention on this group of compounds is justified by their broad health-promoting features. The most commonly known ones are anti-inflammatory, anti-cancer, and antioxidant activities, as well as cancer prevention effects [26]. Especially, the chemopreventive effect of several ITCs, such as sulforaphane, has been extensively studied and attributed to detoxification of the cells (of, e.g., carcinogens) via the glutathione S-transferases (GSTs) facilitated by the ITCs; therefore, several widely promoted health campaigns have pointed to the beneficial effect of increasing consumption of vegetables such as broccoli or cabbage. Equally interesting is anti-inflammatory effect of ITCs, where the compounds exert their effects by modulating cytokine response, affecting toll-like receptors and transcription regulators on multiple levels [27]. Thus, as the currently increasing number of illnesses have been described as related to the general inflammatory response, such a role of widely available phytochemicals is invaluable. Therefore, extensive studies have been conducted to identify new compounds for therapeutical use among this group. Such a search is additionally facilitated by the reports from folk medicine of the beneficial effects of, e.g., mustard seed, horseradish, broccoli, and many others still included in dietary advice. Moreover, the safety of ITCs as a part of the human daily diet

as well as in direct therapy has been assessed, along with the therapeutical doses used in, e.g., the inhibition of cancer cell invasion and angiogenesis, sensitizing cancer cells to chemotherapy, or directing cancer cells to cell cycle arrest, autophagy, and apoptosis. Clinical trials conducted recently have demonstrated the potential of ITCs to protect from or treat serious diseases such as breast, prostate, or lung cancer; inflammatory illnesses; diabetes; and heart disorders [28]. The antimicrobial effects of ITCs have recently attracted increased attention due to the dramatic decline in the introduction of new agents and the limited effectiveness of already existing ones. The effectiveness of ITCs against bacteria have been demonstrated for various species *in vitro*; in addition, a very limited level of microbial resistance to these compounds has been reported [29]. Moreover, clinical trials have documented the effect of ITCs in the treatment of urinary tract and respiratory infections [27], as separate compounds or in a combination of ITCs [30]. Various ITCs exhibit varying antibacterial effects, with phenethyl isothiocyanate being one of the most potent and studied against, e.g., human oral pathogens or bacteria from human guts [31,32]. EHEC strains as the target of ITCs have also been explored, aiming to establish alternative or at least supporting therapy to the risky usage of classical antibiotics. The approaches of employing ITCs against EHEC is described in the following paragraphs.

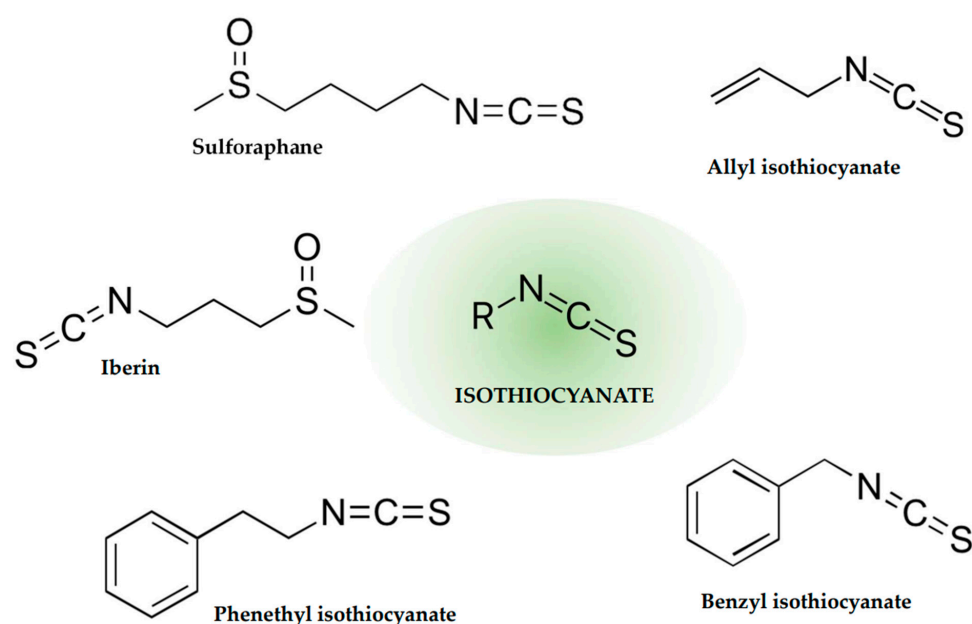


Figure 2. Structural representation of isothiocyanates ($R-N=C=S$) and their key derivatives. In green shade, the general structure of ITC is presented.

3.1.1. Allyl Isothiocyanate

Allyl isothiocyanate (AITC), derived from glucosinolate sinigrin, is responsible for the specific pungent flavor and odor of, e.g., horseradish and mustard. Its antibacterial effect against foodborne pathogenic bacteria has been reported [33], prompting researchers to study the potential use of this ITC in food preservation. It was based on and supported by the traditional use of mustard seeds in long-term preservation of food products (mainly vegetables). AITC use in food preservation has been approved in Japan for over 20 years, providing it comes from a natural source [29]. More detailed studies focused on the specific antimicrobial applications of AITC. As EHEC strains have been often found in processed meat products, especially beef, which is also consumed uncooked or undercooked (e.g., beef tartare or widely eaten hamburgers), special caution has to be taken to prevent spreading the contamination during processing and storage. Other sources of EHEC contamination are vegetables, particularly leafy ones, used in ready-to-eat salads and sprouts. Actually, in

the recent European outbreak of EHEC infection in 2011, which caused an unusual number of complication incidents, the etiological factor was identified as the EHEC O104:H4 strain, present in contaminated sprouted seeds [34]. The use of AITC as an agent preventing bacterial spread in packed food products such as ground beef has been systematically studied, providing satisfactory results of reducing the number of pre-inoculated *E. coli* O157:H7 by at least 3.0 logs with moderate stability of AITC after days of incubation in refrigerated or frozen products [35]. The use of microencapsulated AITC facilitated the handling of AITC but reduced its effectiveness in eliminating pathogenic strains. Interestingly, the number of microbiome-representative bacteria was not significantly altered by AITC [36]. Allyl isothiocyanate has been used also for the protection of vegetable products. For example, AITC has been showed to be an effective agent in eliminating EHEC bacteria from alfalfa seeds, and was then proposed to be employed as a disinfectant instead of as chlorine treatment [37]. The extended delivery of AITC to stored food was achieved by using encapsulation in calcium alginate beads, whose effectiveness was tested on spinach leaves [38]. Another potential application of AITC as a preventive countermeasure against EHEC takes advantage of the combined effect of several natural compounds. For example, a mix of garlic essential oil, AITC, and nisin was employed in the preservation of fresh meat products [39]. The higher effectiveness in microbial control of the mixed compounds (AITC, cinnamaldehyde, and carvacrol) in the vapor phase was shown for EHEC on lettuce and spinach leaves [40].

The proposed mechanism of AITC antibacterial effect in EHEC eradication was discussed to involve metabolic disturbance by inhibition of thioredoxin reductase (necessary in ribonucleotides synthesis) and acetate kinase (used in general energy metabolism) [41]. However, it has also been suggested that AITC affects cell membrane integrity, resulting in leakage of cell content and subsequent cell death [42]. AITC is shown to be active in the range of a minimum inhibitory concentration (MIC) from 100 to 400 µg/mL against *E. coli* O157:H7. The insight into the mechanism of AITC action was provided by the finding that *E. coli* mutants devoid of the BaeSR two-component system exhibit higher resistance to AITC. This system is responsible for controlling the expression of drug-exporting genes and sensing the environmental stress [43].

3.1.2. Phenethyl Isothiocyanate

Phenethyl isothiocyanate is a product of gluconasturtiin hydrolysis, which is abundant in watercress. The antibacterial effect of this compound was shown for several bacterial pathogens causing foodborne diseases, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *E. coli*, and intestinal bacteria such as various clostridia [43,44]. Interestingly, the synergistic effect of PEITC and certain antibiotics was presented for ESBL *E. coli* isolates [45]. The proposed PEITC mode of action suggests a direct effect on membrane integrity. This leads to cytoplasm leakage and K⁺ ion release [45]. The alternative mechanism was shown for EHEC strains by our group, explaining the bacterial growth inhibition by the global stress response directly and indirectly affecting cellular metabolism [46]. This mechanism is discussed in detail in the following chapters. Reported EHEC growth inhibitory PEITC concentrations ranged from 40 to 1000 µg/mL.

3.1.3. Sulforaphane

Sulforaphane (SFN), and its precursor, glucoraphanin, are present in abundance in Brassicaceae plants common in the human diet, such as cabbage, broccoli, or Brussels sprouts. Its bio-active features are widely studied in terms of anticancer and chemopreventive roles, highlighting the SNF-mediated protection at all stages of carcinogenesis [47]. The antibacterial effect of SFN was reported for various respiratory pathogens such as

Streptococcus pneumoniae, *Haemophilus influenzae*, and *S. pyogenes* [48]. The high effectiveness of SFN among other ITCs against enteropathogens, including EHEC (MIC ranging 350–700 µg/mL), prompted further studies encompassing its possible preventive and therapeutic use [31]. The antibacterial effect of SFN against Shiga toxin-producing *E. coli* strains have been studied by us, presenting novel mechanisms of ITC action [49] (see below).

3.1.4. The Induction of the Stringent Response as a Universal Mechanism of ITC Antimicrobial Effect

The controversies and discrepancies among the proposed hypotheses about possible mechanisms of ITC effects and the quite limited number of reports elucidating these mechanisms led to more extensive investigation of this phenomenon—in particular, because of the necessity to fully understand the processes leading to bacterial growth and virulence inhibition before the compounds could be used in therapy. The observation that all tested ITCs varying in side residues (aromatic, aliphatic with various chain length) inhibited the nucleotide synthesis (both DNA and RNA), and in particular, that the production of RNA was dramatically downregulated just after the addition of ITC, prompted the hypothesis that the global regulatory mechanism of the stress response in bacterial cells can be a target of ITCs. The stringent response of the bacterial mechanism to deal with stress and various energy and macromolecules deprivation leads to the inhibition of stable RNA synthesis abruptly after the onset of stress conditions. The alarmones of this stress—unusual guanosine nucleotides, (p)ppGpp—are synthesized by widely conserved enzymes Rel and Spo, or bifunctional RSH [50]. The accumulation of (p)ppGpp was observed in *E. coli* cells treated with ITCs, namely, sulforaphane (SFN), allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), phenyl isothiocyanate (PITC), and isopropyl isothiocyanate (IPRITC) [46,51]. Growth of both wild-type *E. coli* and O157:H7 clinical isolates was inhibited by all tested ITCs, with the lowest MIC for PEITC and BITC. The bacterial growth inhibition was clearly attributed to (p)ppGpp directing the cellular metabolism to energy-saving mode. Interestingly, under this experimental setup, ITCs have not caused membrane disruption, which contradicts previous reports, nor did it increase reactive oxygen species formation. The latter finding is important in the light of the risk of DNA damage resulting in prophage induction and Shiga toxin synthesis. The most important, though, was finding that ITCs, while inhibiting bacterial growth, did not prompt *stx* gene expression [46,51], which was due to the efficient inhibition of prophage induction, with the main target being the transcription from early phage promoters such as pR. The impairment of phage lytic development under ITC treatment was observed even in the presence of the DNA damaging agent mitomycin C. As a result, the bacterial lysates treated with ITCs decreased significantly their virulence against HeLa and Vero cells. All above-mentioned effects were observed only when (p)ppGpp accumulated, and the effects were absent in mutants devoid of the functional RelA enzyme. RelA is responsive to amino acid starvation; thus, the hypothesis was proposed that ITCs induce a stringent response by blocking protein synthesis either by direct interaction with amino acids (which was previously shown for AITC) or by the impairment of tRNA aminoacyl transferase or synthetase. This is supported by the finding that an excess of certain amino acids reversed the antibacterial effect of ITCs. Further work on various aliphatic ITCs, such as SFN, sulforaphane (SFE), iberiverin (IBR), iberin (IBN), allysin (ALN), erucin (ERU), erysolin (ERY), and cheirolin (CHE), support this hypothesis, as all tested compounds induced the stringent response, causing bacterial growth and prophage induction inhibition in various tested clinical isolates of *E. coli* O157:H7. Interestingly, a synergistic effect was observed for some ITC combinations [52].

The finding of the apparently common mechanism of ITC action on EHEC strains provides very interesting input in the debate on the treatment of EHEC infections. First,

ITCs can be considered an option of direct therapeutical agents, especially due to previous extensive studies on their safe usage. Then, the specific mechanism of ITC action can be employed in combined therapy with ITCs and antibiotics to reduce or fully exclude the risk of phage lytic development induction and subsequent Shiga toxin synthesis.

3.2. Phenolic Compounds

Common phenolic compounds include (i) polyphenols, such as lignin and tannins; (ii) oligophenols, such as flavonoids, stilbenes, and coumarins; and (iii) monophenols (simple phenolic acids), such as benzoic acid derivatives (hydroxybenzoic acids) and cinnamic acid derivatives (hydroxycinnamic acids). Phenols and polyphenols are a diverse group of chemical compounds characterized by the presence of at least one aromatic ring and hydroxyl groups (Figure 3). Based on the number and arrangement of carbon atoms, they are classified into 10 major classes [53,54]. This structural diversity translates into a broad spectrum of biological functions. To date, more than 8000 polyphenols have been identified, known not only for their potent antioxidant properties but also for their antimicrobial, antifungal, and antibacterial activities [55,56]. In addition to their direct impact on metabolic pathways, polyphenols also exert anti-inflammatory effects by downregulating production of pro-inflammatory cytokines and modulating immune responses. Their antioxidant potential extends to protecting cells from oxidative damage, which is linked to a reduction in the risk of neurodegenerative disorders such as Alzheimer's and Parkinson's disease and the prevention of cardiovascular diseases and cancers [57]. Recent studies have also explored their role in improving gut health, as polyphenols can influence the composition of the intestinal microbiota, promoting beneficial bacteria and suppressing pathogenic strains [58]. Furthermore, they have the ability to modulate various signaling pathways, including the regulation of cytochrome P450 enzyme activity, which influences drug metabolism [59,60]. For example, epigallocatechin gallate (EGCg) inhibits the activity of cytochrome P450 enzymes, while bergamottin, a natural compound found in grapefruit, inhibits the CYP2C19 isoenzyme, affecting detoxification mechanisms. A significant part of the research explores the impact of polyphenols on pathogenic *E. coli*, particularly enterohemorrhagic strains (as shown in Table 1).

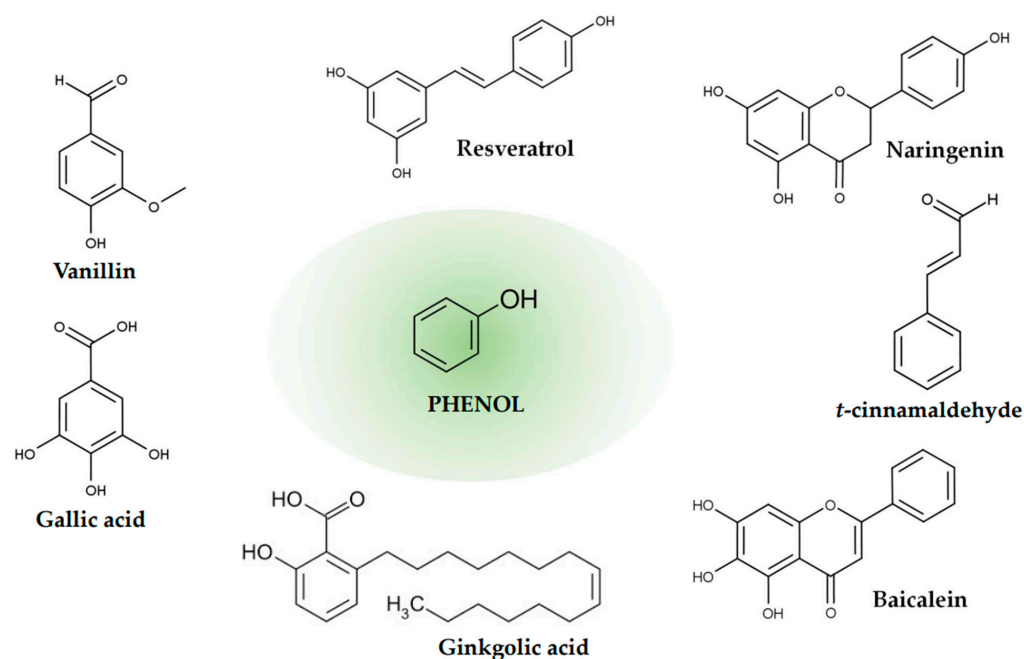


Figure 3. Structural representation of phenolic compounds and their derivatives. The central phenol structure (green-shaded area) highlights the core functional group present in all depicted molecules.

Table 1. Anti-EHEC activity of phenolic compounds.

Compound Type	Compound	Origin	Concentration	Mechanism/Effects on EHEC	Reference
Flavonoids	Apigenin	Citrus fruit	Not specified	Inhibits biofilm formation by suppressing QS signals	[61]
	Baicalein/baicalin	Chinese skullcap (<i>Scutellaria baicalensis</i> Georgi)	0.13 mmol/L	Inhibits Stx2 oligomer formation, reducing cytotoxicity and renal damage; suppresses virulence gene expression.	[62–64]
	Quercetin	Caper (<i>Capparis</i> sp.), onions (<i>Allium cepa</i> L.), kale (<i>Brassica oleracea acephala</i>)	≤1.4 g/L 32 µg/mL	Suppresses biofilm formation and virulence through inhibition of <i>luxS</i> and <i>pfs</i>	[61,65,66]
	Kaempferol	Citrus fruit, various plants	100 µg/mL 32 µg/mL	Inhibits growth and biofilm formation by QS modulation	[61]
	Naringenin	Citrus fruits	7.3–11.0 mM	Inhibits biofilm formation, nonspecific quorum-sensing inhibitor	[61,67]
	Chrysoeriol, sudachitin, acacetin	<i>Chrysanthemum lavandulifolium</i>	10 mg/mL	Downregulates <i>stx1A</i> and <i>stx1B</i> gene expression, reducing toxin synthesis	[68]
	Catechins (e.g., epigallocatechin)	Green tea (<i>Camellia sinensis</i> L.)	5 mg/mL	Reduced <i>ompC</i> and <i>rpoS</i> expression, inhibition of ROS dysmutase activity	[69–71]
Phenolic acids	Gallic acid	Grains, tea (<i>Camellia sinensis</i> L.)	1.0–5 mg/mL	Inhibits swarming motility, biofilm formation, and quorum sensing (<i>csgA</i> , <i>fimA</i> , <i>eae</i> genes)	[72–74]
	Methyl gallate	Not specified	0.02–0.4 mg/mL	Overexpressed <i>csgA</i> and <i>cyaA</i> without increasing biofilm, reduced motility by 41.8% at 0.05 mg/mL	[73]
	Protocatechuic acid	Various plants	MIC 2.5 mg/mL	Disrupted cell membranes, increasing permeability, synergistic with high hydrostatic pressure	[75]
	Vanillic acid	Grains, fruits, herbs	10 mM	5.0 log reduction in bacterial populations after 7 days	[76]
	Caffeic acid	Apple (<i>Malus domestica</i> Borkh.)	0.2 mg/mL	Reduced viable counts below detection levels at pH 3.2	[77]
	p-Coumaric acid	Peanuts (<i>Arachis hypogaea</i> L.), beans (<i>Phaseolus vulgaris</i> L.), tomatoes (<i>Solanum lycopersicum</i> L.)	0.5%	Increased <i>E. coli</i> O157:H7 death rate by 23-fold	[78]

Table 1. Cont.

Compound Type	Compound	Origin	Concentration	Mechanism/Effects on EHEC	Reference
Phenylpropanoids	Coumarin and derivatives	Cinnamon (<i>Cinnamomum verum</i> J. Presl), mint (<i>Mentha × piperita</i> L.), green tea (<i>Camellia sinensis</i> L. Kuntze), lavender (<i>Lavandula angustifolia</i> Mill.)	50 µg/mL	Inhibited biofilm formation >80%, repressed curli and motility genes, esculetin repressed Shiga-like toxin II gene	[79,80]
	<i>t</i> -Cinnamaldehyde	Cinnamon (<i>Cinnamomum verum</i> J. Presl, <i>Cinnamomum cassia</i> L. J. Presl)	MIC 1.875–3.750 mM	Inhibition of bacterial growth by the induction of the stringent response, blocking of phage lytic cycle and Shiga toxin production	[81–86]
	Ginkgolic acid	Ginkgo (<i>Ginkgo biloba</i> L.)	1–5 µg/mL	Inhibited biofilm formation of up to 70% at 1 µg/mL and nearly abolished at 5 µg/mL, reduced fimbriae, altered motility, suppressed curli expression	[87,88]
	β-Resorcylic Acid	Various angiosperms	1%	Reduces bacterial population on surfaces by disrupting biofilm formation and cell adhesion	[89]
Stilbens	Resveratrol and derivatives	Grapes (<i>Vitis vinifera</i> L.), red wine, peanuts (<i>Arachis hypogaea</i> L.),	8.7–13.0 mM	Represses motility and biofilm-related genes (<i>flh</i> , <i>csg</i> , <i>fim</i> , <i>motB</i>), inhibits QS pathways	[67,90–92]
Other	Eugenol	Basil (<i>Ocimum basilicum</i> L.), cinnamon (<i>Cinnamomum verum</i> J. Presl), cloves (<i>Syzygium aromaticum</i> (L.) Merr. & Perry)	0.5% <i>w/w</i> in food preservation	Increases membrane permeability, inhibits biofilm formation through downregulation of <i>csg</i> and <i>fim</i> genes	[93–97]
	Phloretin	Apple (<i>Malus domestica</i> Borkh.)	25–50 µg/mL	Reduced biofilm formation; repressed toxin genes (e.g., <i>hlyE</i> , <i>stx2</i>), autoinducer-2 importer genes, curli genes; reduced fimbriae production	[98]
	Propolin D	Parasol leaf tree (<i>Macaranga tanarius</i> (L.) Müll.Arg.)	10 to 50 µg/ml	Disrupts biofilm integrity by inhibiting <i>csgA</i> and <i>csgB</i> expression, reducing curli production	[99]
	Total blueberry phenolics	Wild blueberries (<i>Vaccinium myrtillus</i> L.)	0.8–1.8 g/L	Increased cell membrane permeability, leading to CFU reductions of 6.0–7.0 log for various fractions like MPA, A&P, and anthocyanins	[100]

3.2.1. Flavonoids

Among the polyphenols, flavonoids have been particularly studied for their biofilm-inhibiting effects. Jin-Hyung Lee and colleagues tested nine flavonoids for their impact on *E. coli* O157:H7 biofilm formation. The study also included antioxidant compounds such as vitamins C and E, which did not affect biofilm formation, suggesting that the biofilm-inhibiting properties of flavonoids were not related to their antioxidant activity. Phloretin emerged as the most potent flavonoid, reducing EHEC biofilm formation by 89% and 93% at concentrations of 25 and 50 µg/mL, respectively. Interestingly, phloretin did not inhibit biofilm formation in four strains of *E. coli* K-12 and non-pathogenic *E. coli* strains; in fact, biofilm formation was enhanced. These findings were supported by qRT-PCR analysis, which showed that genes repressed in the biofilm cells of *E. coli* O157:H7 (*hlyE*, *lsrA*, *lsrB*, *csgA*, and *csgB*) were strongly induced in the biofilm cells of *E. coli* K-12. DNA microarray data also indicated that phloretin inhibited the expression of Shiga toxin 2 (Stx2) genes. Importantly, their results confirmed that phloretin exhibited anti-inflammatory properties in both in vitro and in vivo models of colitis.

Vikrama et al. [61] studied several other flavonoids, including naringenin, kaempferol, quercetin, and apigenin. These compounds inhibited the activity of the AI-2 signaling molecule in *Vibrio harveyi*, indicating that they are intercellular signaling inhibitors (autoinducers), which are critical in biofilm formation. These flavonoids also reduced biofilm formation by *V. harveyi* and *E. coli* O157:H7.

As demonstrated above, the beneficial effects of polyphenols on EHEC do not necessarily rely solely on their bactericidal action. Another potential mechanism exhibited by polyphenols is their ability to bind directly to Shiga toxin, thereby inactivating it. Dong et al. [63] demonstrated that baicalin (BAI), a flavonoid isolated from *Scutellaria macrantha* Fisch., induces oligomer formation in the toxin through direct binding. In vitro studies using HeLa cells showed that when BAI was either mixed with rStx2 prior to cell addition or incubated with HeLa cells treated with 50 pg/mL rStx2, it significantly protected the cells at concentrations of 4.5 µM, with maximum protection observed at 36 µM. Importantly, in a mouse model, BAI administration (100 mg/kg dose) every 6 h for a total of 23 doses following rStx2 exposure provided approximately 70% protection by day 6. Mice that did not receive BAI treatment showed significant kidney damage, including tubular swelling and hyaline casts, while kidneys in BAI-treated mice appeared similar to those of control animals not exposed to the toxin. Further efficacy of BAI against *E. coli* O157:H7 infections was demonstrated in both tissue culture and mouse models [62]. In tissue culture, BAI addition protected HeLa cells from Stx-induced damage, with significant protection at 9 µM and maximum protection at 72 µM. In a mouse model, BAI co-inoculation with *E. coli* O157:H7 and mitomycin C (which induced 100% mortality within 8 days in untreated, infected animals) reduced mortality to 20% on day 8, thus providing approximately 80% protection. Histopathological examination revealed that untreated, infected mice showed kidney damage marked by swelling, discoloration, and necrosis, while the BAI-treated group showed significantly reduced damage. Another study by Vinh et al. investigated baicalein, a structural analogue of BAI [64]. When baicalein was pre-incubated with Shiga toxin and then added to Vero cell cultures, Vero cell viability decreased in response to increasing concentrations of Stx1 and Stx2. However, baicalein pre-treatment significantly reduced Stx1 and Stx2 cytotoxicity, protecting Vero cells by binding to the surface of their cytoplasmic membranes and altering membrane function. At a concentration of 0.13 mmol/L, pre-incubation with baicalein decreased Vero cell sensitivity to Stx1 and Stx2 by approximately 6- and 8-fold, respectively. Molecular docking analysis suggested that baicalein forms a complex with both Stx1B and Stx2B pentamers. However, in cellular models, Stx binding to the Vero cell surface was preferred over baicalein binding to StxB pentamers,

which aligns with the observed results. Interestingly, gene expression analysis revealed that baicalein slightly increased the transcription of *stx1* but not *stx2*, compared to a negative control [64].

Additionally, baicalin and baicalein exhibit a range of pharmacological activities beyond their effects on Shiga toxin. These compounds help reducing inflammation during bacterial infections by scavenging reactive oxygen species (ROS) [101]. Baicalin also synergizes with various antibiotics by disrupting bacterial tolerance mechanisms, enhancing bactericidal effects through membrane disruption, efflux pump inhibition, and biofilm formation reduction [102]. Interestingly, baicalin shows antimicrobial activity against *Helicobacter pylori* but does not affect probiotic strains, and it even promotes biomass growth in *Lactobacillus casei* [103].

3.2.2. Resveratrol

Resveratrol, a polyphenolic stilbene compound found in foods such as red grapes and berries, is largely known for its health benefits as an antioxidant [104,105]. However, recent studies have also highlighted its significant antimicrobial activity against EHEC [67]. Lee et al. demonstrated antibiofilm properties of resveratrol toward EHEC [91]. Transcriptional analyses showed that the extract of *Carex dimorpholepis* Steud. repressed curli genes, various motility genes, and AI-2 quorum-sensing genes, which was corroborated by a reduction in the production of fimbria, motility, and biofilm by EHEC. Trans-resveratrol at 10 µg/mL in the extract of *C. dimorpholepis* was found to be a potent anti-biofilm compound against EHEC. Such biofilm inhibition was not limited to resveratrol alone; its oligomeric derivatives, including dimers and tetramers, showed even stronger anti-biofilm activity [90,92]. Furthermore, resveratrol-enriched extract was found to significantly decrease the adhesion of EHEC to human epithelial cells without compromising host cell viability. This dual action—reducing pathogen adhesion and maintaining host cell health—highlights resveratrol's potential as a safe and effective antimicrobial agent in preventing EHEC infections [91]. These findings underscore the potential of resveratrol and its derivatives as natural antimicrobials in both clinical and food safety applications, providing a multifaceted approach to combating EHEC.

3.2.3. Cinnamaldehyde

Cinnamaldehyde (*t*-CA), also known as 3-phenyl-2-propenal, is an unsaturated aldehyde belonging to the phenylpropanoid class. The history of use of cinnamon (with *t*-CA as its main content) reaches ancient times and included various areas and continents; in its use, the antimicrobial and preservation-promoting features of cinnamon have been utilized. It involved the application of cinnamon as a spice, in religious ceremonies, in fragrances, as a digestive help, and as an appetite stimulant. Currently, *t*-CA is widely used mostly as a spice and thus is considered a safe daily diet component. The beneficial health role of *t*-CA includes, but is not limited, to its antioxidant, anti-inflammatory, and anti-diabetic action [106]. Research indicates that *t*-CA affects membrane potential and permeability, leading to protein oxidation, cytoplasmic leakage, and acidification, thereby reducing the toxicity of EHEC strains [86]. Additional studies have shown that sublethal concentrations of *t*-CA modulate the expression of virulence genes in *E. coli* O157:H7 and reduce bacterial motility (e.g., *fliA* and *motA* genes) [85]. An in-depth investigation into the mechanism of cinnamaldehyde's action against EHEC strains revealed that reduced virulence is associated with the suppression of Stx prophage induction and Shiga toxin expression. This is mediated through the stringent response, and its alarmone molecules (p)ppGpp, which is activated by *t*-CA [106]. The study from our lab shows that the activity of *t*-CA led to amino acid depletion and thereby cell starvation by triggering acidic stress conditions. We showed

in the study that *t*-CA activity led to impairment in metabolic processes as a tricarboxylic acid (TCA) cycle and energy-consuming processes like flagellar assembly. We assumed the observed effects were consequences of global gene expression regulation mediated by (p)ppGpp. Importantly, alarmones of the stringent response are the inhibition factors of the major lytic promoter (pR) of lambdoid phages [106]. Mechanistically, *t*-CA activity disrupts intracellular pH homeostasis, prompting the bacterial cell to utilize amino acids such as L-lysine and L-serine as part of its defense mechanisms. Consequently, this leads to depletion of these key amino acids and activation of RelA-dependent alarmone production. This cascade of events not only impacts bacterial metabolism but also compromises the ability of the pathogen to sustain its virulence. Numerous studies have investigated *t*-CA applications in the food industry, where it is particularly valued for its antimicrobial properties. It has demonstrated potential efficacy against various strains of *S. aureus*, *Salmonella* spp., *Bacillus* spp., and *E. coli*, including EHEC strains [83,84]. For example, a study by Manu et al. showed that cinnamaldehyde (1.5 µL/mL) at 4 °C completely inactivated *E. coli* O157:H7 inoculated in carrot juice within 8 h [82]. Similarly, Baskaran et al. reported that at 4 °C, cinnamaldehyde concentrations of 0.125% and 0.075% *v/v* reduced bacterial counts in apple juice and cider to undetectable levels by days 3 and 5 of the experiment, respectively [81]. EHEC strains are frequently detected on vegetables; however, treatment of leafy greens with *t*-CA has been shown to effectively reduce these pathogens in an environmentally friendly manner [81].

3.2.4. Other Phenolics

While the antimicrobial activity of many natural phenolics has been established, studies aimed at implementing such compounds against EHEC are especially valuable when they extend beyond in vitro tests and use animal models, as the effects of the compounds in such systems may differ from expected outcomes. For example, Kang-Mu Lee and colleagues [107] confirmed the inhibitory effect of epigallocatechin gallate (EGCg) from green tea on the virulence of EHEC strains using the *Caenorhabditis elegans* model. The nematodes were exposed to the pathogen with and without EGCg. After 5 days, survival rates of *C. elegans* were 47.1% without EGCg and 76% with EGCg. Furthermore, the researchers observed a reduction in the transcription of several virulence genes (*eae*, *escN*, *espA*, *sepZ*, and *tir*), regulated by quorum sensing at a concentration of 25 µg/mL—lower than the MIC (539 ± 22 µg/mL) they determined. The transcription levels of these genes decreased by 72.1%, 87.3%, 79.5%, 78.8%, and 90.8%, respectively.

The same model was used to investigate the effects of other compounds, such as coumarin, esculetin, and umbelliferone [80]. Esculetin demonstrated a dose-dependent effect on the survival of infected *C. elegans*, which corresponded to the repression of the *stx2* gene observed in qRT-PCR experiments. However, despite showing antibacterial properties against EHEC, neither coumarin nor umbelliferone affected the survival of *C. elegans* or the expression of Shiga toxin genes. Interestingly, both coumarin and umbelliferone, at concentrations of up to 50 µg/mL, exhibited high anti-biofilm activity without inhibiting planktonic cell growth at the same concentration. This indicates that the biofilm reduction is associated with an anti-biofilm mechanism rather than bacterial growth inhibition.

These studies confirm that, due to the virulence mechanisms of EHEC strains, simply determining the antibacterial properties of a compound is not sufficient. It is essential to thoroughly investigate the mechanisms of their action. Overall, studies on the impact of polyphenolic compounds on EHEC bacteria have often focused on their anti-biofilm properties. While *E. coli* O157:H7 is non-invasive and poorly forms biofilms, rare O157 strains that produce curli exhibit strong biofilm formation at 37 °C and possess the ability to invade epithelial cells. This correlates with a single-base-pair transversion (A to T)

in the curli promoter [18]. Additionally, biofilm formation can be crucial in terms of its development on food products, which are known to be carriers of EHEC and may serve as a source of infection [108,109].

Other studies [110] focused on the binding of compounds to the toxin, investigating galloylated catechins (GCg) and epigallocatechin gallate (EGCg). Both compounds inhibited Stx1 cytotoxicity in Vero cells after pre-incubation at a concentration of 100 mg/L but did not show the same activity against Stx2. In silico studies indicated that EGCg formed a more stable structure with the Stx1B pentamer than with the Stx2B pentamer.

The use of polyphenols as natural preservatives in food processing is gaining increasing attention. Numerous studies highlight their antibacterial properties, demonstrating their ability to effectively combat both Gram-positive and Gram-negative bacteria. Additionally, some polyphenols exhibit synergistic effects when used in combination with traditional food preservatives or processing methods [53]. In a study by Wu et al., it was found that cranberry concentrate, containing high levels of polyphenols, at concentrations of 75 µL/mL and 100 µL/mL reduced *E. coli* O157:H7 by 0.8 log and 3.0 log CFU/mL, respectively, compared to the control after 24 h [111]. Additionally, cranberry concentrate (at 2.5%, 5%, and 7.5% *w/w*) reduced the number of *E. coli* O157:H7 inoculated into ground beef by 0.4 log, 0.7 log, and 2.4 log CFU/g, respectively, compared to the control on day 5 [112]. To further investigate the direct effect of polyphenols on EHEC, another study fractionated cranberry extract to isolate its polyphenolic components and evaluated their impact independently. Results showed that the polyphenol fractions reduced the bacterial count to below detectable levels at concentrations of 5.40 g/L and 2.70 g/L compared to the control. *E. coli* O157:H7 cells treated with polyphenols derived from *Vaccinium macrocarpon* Aiton (cranberry) exhibited localized damage to the outer membrane, cytoplasmic leakage, and irregular cell shape [113].

3.3. Terpenes and Terpenoids

Terpenes and terpenoids are naturally occurring compounds found in various plants, including fruits, vegetables, and herbs. They contribute to the characteristic flavors and aromas of these plants and often have therapeutic properties [114]. Some terpenes and terpenoids have been studied for their potential antibacterial properties, including their effects against pathogenic bacteria such as *E. coli*, and specifically Shiga toxigenic strains [40,115]. Terpenes are hydrocarbons derived from the combination of isoprene units (C₅) (Figure 4), while terpenoids are modified terpenes that have undergone oxidation or other chemical alterations. Both terpenes and terpenoids can exhibit antimicrobial activity through various mechanisms, including disrupting bacterial cell membranes, interfering with cell wall synthesis, and inhibiting essential enzymes [114–116].

Several studies have investigated the antibacterial effects of terpenes and terpenoids against *E. coli*, including EHEC (as shown in Table 2).

Table 2. Plant-derived terpenes and terpenoids targeting EHEC virulence factors.

Compound	Origin	Concentration	Mechanism/Effects on EHEC	Reference
Carvacrol	Oregano (<i>Origanum vulgare</i> L.), thyme (<i>Thymus vulgaris</i> L.)	MIC 0.0005–0.310 mg/mL	Disrupts cell membranes, interferes with enzyme systems, downregulates virulence genes, inhibits biofilm formation	[40,94,117–124]
Thymol	Oregano (<i>Origanum vulgare</i> L.), thyme (<i>Thymus vulgaris</i> L.)	MIC 0.0001–0.310 mg/mL	Disrupts bacterial membranes, reduces motility, decreases biofilm formation, downregulates virulence genes	[94,118,119,121,125–127]

Table 2. Cont.

Compound	Origin	Concentration	Mechanism/Effects on EHEC	Reference
Menthol	Mint (<i>Mentha piperita</i> L., <i>Mentha spicata</i> L.)	MIC 0.0001–11 mg/mL	Disrupts bacterial cell membranes, reduces biofilm formation, decreases expression of virulence gene	[119,128,129]
Camphor	Cinnamon (<i>Cinnamomum camphora</i> Ness et Eberm.), thyme (<i>Thymus vulgaris</i> L.), lavender (<i>Lavandula angustifolia</i> Mill.), basil (<i>Ocimum basilicum</i> L.), sage (<i>Salvia officinalis</i> L.)	MIC 0.008 mg/mL	Disrupts cell membranes, interferes with metabolic functions	[119,130]
Alpha/beta-pinene	Mint (<i>Mentha spicata</i> L.), lemon (<i>Citrus limon</i> Burm.), sage (<i>Salvia officinalis</i> L.), thyme (<i>Thymus vulgaris</i> L.)	MIC 0.008 mg/mL	Inhibits microbial cell membrane integrity	[118,119]
Linalool	Lavender (<i>Lavandula angustifolia</i> Mill.), basil (<i>Ocimum basilicum</i> L.), mint (<i>Mentha piperita</i> L.), chamomile (<i>Matricaria chamomilla</i> L.), thyme (<i>Thymus vulgaris</i> L.)	MIC 0.006 mg/mL	Disrupts bacterial cell membranes, interferes with membrane permeability, inhibits bacterial enzymes, disrupts biofilm formation	[119]
Eucalyptol (1,8-cineole)	Mint (<i>Mentha spicata</i> L., <i>Mentha piperita</i> L.), chamomille (<i>Matricaria chamomilla</i> L.), lavender (<i>Lavandula angustifolia</i> Mill.), basil (<i>Ocimum basilicum</i> L.), sage (<i>Salvia officinalis</i> L.), thyme (<i>Thymus vulgaris</i> L.)	MIC 0.006–0.032 mg/mL	High water solubility enhances diffusion in agar media, elongates bacterial lag phase	[119,131,132]
α -terpineol	White jade orchid tree (<i>Michelia</i> $\times$ <i>alba</i> DC.)	MIC 2 mg/mL	Disrupts bacterial cell membranes, affects ATP leakage and membrane fluidity	[133–135]
Geraniol	Basil (<i>Ocimum basilicum</i> L.)	MIC 3 mg/mL	Inhibits glucosyltransferase, affecting biofilm formation	[136,137]
Limonene	Mint (<i>Mentha spicata</i> L., <i>Mentha piperita</i> L.), lemon (<i>Citrus limon</i> Burm., <i>Citrus aurantium</i> L.), chamomille (<i>Matricaria chamomilla</i> L.), lavender (<i>Lavandula angustifolia</i> Mill.), basil (<i>Ocimum basilicum</i> L.), sage (<i>Salvia officinalis</i> L.), thyme (<i>Thymus vulgaris</i> L.)	MIC 0.01 mg/mL	Disrupts cell membranes, causes leakage of intracellular contents, interacts with DNA, unwinds plasmids	[94,119,134]
Isolimononic acid	Bitter orange (<i>Citrus</i> $\times$ <i>aurantium</i> L.)	IC25 19.7 μ M	Inhibits biofilm formation and attachment, disrupts TTSS and AI-3/epinephrine signaling	[138,139]

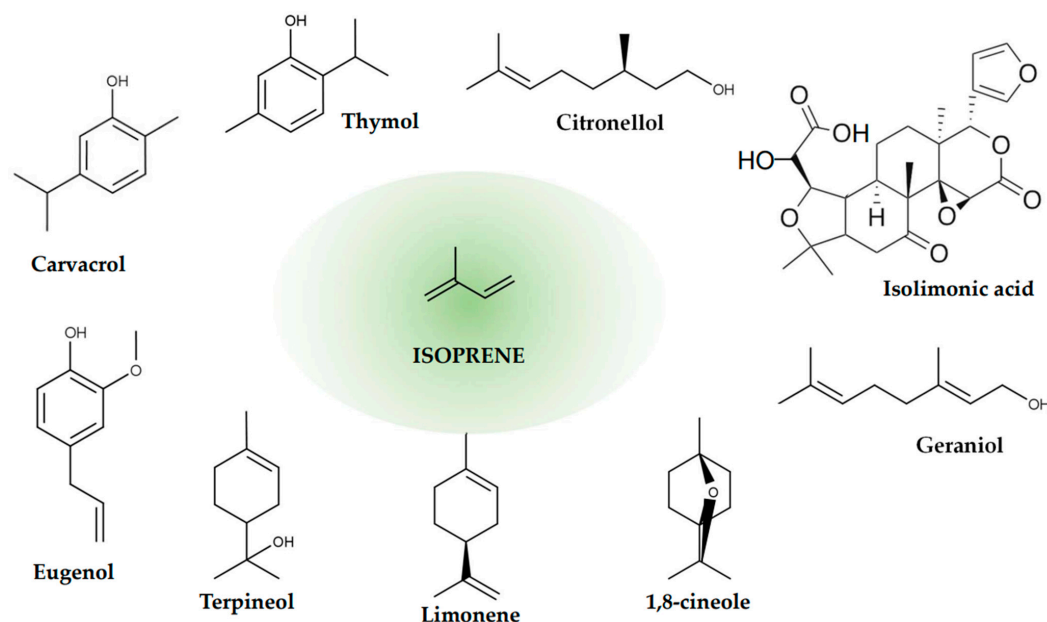


Figure 4. Structural representation of plant-derived terpene derivatives. The central isoprene unit (highlighted in green) serves as the core building block of terpenes and terpenoids.

3.3.1. Carvacrol

Carvacrol is a natural monoterpene phenol. It is a constituent found in the essential oils (EOs) of several aromatic plants, with oregano (*Origanum vulgare* L.) and thyme (*Thymus*

vulgaris L.) being notable sources. The essential oils extracted from these plants have been used for centuries in traditional medicine and culinary applications. The chemical structure of carvacrol consists of a phenol group (a hydroxyl group attached to an aromatic ring) and a monoterpene backbone. Its molecular formula is $C_{10}H_{14}O$, and it has the IUPAC name 2-methyl-5-(1-methylethyl)phenol [140]. Carvacrol has gained attention for its potential health benefits, particularly its antimicrobial properties. Studies have suggested that carvacrol exhibits antibacterial, antifungal, and antiviral activities. It may disrupt the cell membranes of microorganisms, interfere with their enzyme systems, and contribute to their overall antimicrobial effects [140,141].

Carvacrol's efficacy against *E. coli* O157:H7 was investigated across various vegetables. The experiment by Landau and Shapira from 2012 involved exposing the bacteria to different concentrations of carvacrol in the vapor phase within a sealed container at temperatures of 0, 4, and 10 °C. On the intact surface of lettuce, even the lowest concentration of carvacrol vapor exhibited efficacy, deactivating over 4.0 log of *E. coli* O157:H7 within 4 days at 0 and 4 °C, and within 2 days at 10 °C. In contrast, on damaged tissue resulting from cutting, the highest concentration demonstrated a substantial reduction in the pathogen population by 4.0 log at 0 °C and 2.0 to 4.0 log at 4 °C over a 4-day period. At 10 °C, these concentrations also achieved a notable decrease in the pathogen population by 1 to 3.0 log within 2 days. Interestingly, the study revealed a more pronounced inactivation effect on lettuce compared to spinach leaves and on leaf surfaces compared to damaged areas in general. This highlights the impact of carvacrol concentration and temperature on different vegetable types and conditions. The findings suggest that utilizing antimicrobials in the vapor phase, specifically employing carvacrol, holds promise for enhancing the safety of refrigerated leafy greens when sold in sealed packaging. This approach could potentially contribute to mitigating the risk of *E. coli* contamination in such food products [129]. Another study by Mith et al. from 2015 investigated the impact of carvacrol on the expression of virulence-associated genes in *E. coli* O157:H7 (ATCC strain 35150), and the study revealed notable effects. Carvacrol exhibited effectiveness by firstly inhibiting the transcription of the *ler* gene, crucial for the upregulation of LEE2, LEE3, and LEE4 promoters, as well as the formation of attaching and effacing lesions. Secondly, it demonstrated a reduction in the expression of both Shiga toxin and *fliC* genes. Additionally, a discernible decrease in *luxS* gene transcription, associated with quorum sensing, was observed. These effects were found to be dose-dependent, highlighting a specific capability of carvacrol to downregulate the expression of virulence genes in EHEC O157:H7. These outcomes suggest that carvacrol holds potential to alleviate the adverse health consequences associated with virulence gene expression in EHEC O157:H7. Considering these findings, the application of carvacrol as a natural antibacterial additive in food or as an alternative to antibiotics emerges as a promising avenue for mitigating the impact of virulence in EHEC O157:H7 [120]. A study by Burt et al. (2005) examined carvacrol bacteriostatic and bactericidal properties with MICs of 1.2 mmol/L and additive in combination. Growth curves in the presence of nonlethal concentrations of carvacrol with the addition of agar (0.05%, *w/v*) or carrageenan (0.125%, *w/v*) as a stabilizer were produced by optical density measurement. The stabilizers agar and carrageenan significantly improved the effectiveness of carvacrol in broth, possibly because of a delay in the separation of the hydrophobic substrate from the aqueous phase of the medium. When carvacrol was dissolved in ethanol before being added to broth, stabilizers were not needed. Carvacrol and thymol, particularly when used in combination with a stabilizer or in an ethanol solution, may be effective in reducing the number of or preventing the growth of *E. coli* O157:H7 in liquid foods [121].

Another study focused on the determination of alteration in the membrane fatty acid profile as an adaptive mechanism of the cells in the presence of a sublethal concentration

of an antimicrobial compound in response to a stress condition. Methanolic solutions of carvacrol were added to growth media of *E. coli* O157:H7 (ATCC 43888). The total percentage of unsaturated fatty acids was slightly increased [94]. Another study provides input on the mechanism of action of carvacrol. Namely, in the presence of 1 mM carvacrol during overnight incubation, bacteria produced significant amounts of heat shock protein 60 (HSP60) (GroEL) ($p < 0.05$) and significantly inhibited the synthesis of flagellin ($p < 0.001$), causing cells to be aflagellate and therefore nonmotile [142]. A study by Baskaran et al. investigated the effect of subinhibitory concentrations of carvacrol on production and virulence gene expression. Carvacrol reduced EHEC motility and attachment to human intestinal epithelial cells ($p < 0.05$) and decreased toxin synthesis by EHEC. The RT-PCR data revealed decreased expression of critical virulence genes in EHEC ($p < 0.05$). The results collectively suggest that carvacrol could be used to reduce EHEC virulence [143].

Carvacrol has undergone testing across various food products, including meat. The process of heating meat at high temperatures or for extended periods to eliminate foodborne pathogens can lead to the formation of potentially carcinogenic heterocyclic amines. To address this issue, ground beef was supplemented with 1% carvacrol, thoroughly mixed, and then inoculated with *E. coli* O157:H7. Subsequently, beef patties were prepared and compared to controls, resulting in a reduction in *E. coli* O157:H7 population of 2.5–5.0 log. The most significant reductions in three major amines, MeIQ (58%), MeIQx (72%), and PhIP (78%), were observed. These findings demonstrate that carvacrol effectively reduced both bacterial count and amine amount in a commonly consumed meat product [144]. Further research indicated that achieving a 4.0 log reduction in contaminated processed ground beef requires heating to an internal temperature of 60 °C for at least 30 min. The sensitivity to heat significantly increased ($p < 0.05$) with the addition of and/or increasing levels of carvacrol from 0.5 to 1.0%. These data can help in establishing critical control points for ground beef with lower heating times and temperatures [117].

Another study explored storage conditions and physicochemical parameters. Beef slices inoculated with *E. coli* O157:H7 were marinated in teriyaki sauce with or without 0.3–0.5% carvacrol. After 1, 3, and 7 days at 4 °C, the indigenous microflora population, color, lipid oxidation, marinade uptake, and pH of the marinated beef and leftover marinade samples were assessed. While teriyaki sauce alone did not reduce or inhibit bacteria, teriyaki sauce containing 0.5% carvacrol inactivated all inocula without recovery within 7 days ($p < 0.05$). Physicochemical parameters were not significantly affected ($p < 0.05$) [145]. In 2016, Moon and Rhee investigated the synergistic effect of soy sauce and carvacrol. Carvacrol (1 mM) eradicated all test bacteria within 1–5 min at 22 °C and within 10 min at 4 °C. The results suggest that carvacrol synergizes with other components present in soy sauce, possibly due to the combination of factors such as high salt concentration and low pH imparted by organic acids in soy sauce, along with the membrane-attacking properties of carvacrol [146]. Another team studied the edible film of *Gelidium corneum* (Clemente Thuret) containing carvacrol as an antimicrobial and antioxidative agent. Increasing amounts of the antimicrobial agent enhanced its activity against *E. coli* O157:H7. Application of the film to ham packaging effectively inhibited microbial growth and lipid oxidation during storage [147].

Due to these properties, carvacrol has been investigated for its potential in food preservation, as well as in the development of natural antimicrobial agents. It is important to note that while carvacrol looks promising, further research is needed to fully understand its mechanisms of action and its applications in various fields. Additionally, the concentration and formulation of carvacrol can influence its efficacy and safety [140].

3.3.2. Thymol

Thymol is a natural monoterpene phenol that is found in several plants, with thyme (*Thymus vulgaris* L.) being one of the most notable sources. It is widely recognized for its strong, aromatic odor and flavor, which makes it a popular ingredient in various culinary and medicinal preparations [148,149].

The impact of pH, temperature, water activity, sodium chloride levels, inoculum size, and the presence of competing microorganisms on thymol's effectiveness against *E. coli* O157:H7 was investigated. It was observed that the susceptibility of *E. coli* O157:H7 to thymol increased as storage temperature, water activity, pH, and inoculum size decreased. Sodium chloride concentrations ranging from 0.5% to 2.5% and the presence of a microflora mixture did not significantly alter thymol antimicrobial effects on *E. coli* O157:H7 ($p < 0.05$). The MIC ranged between 500 and 1000 $\mu\text{g/mL}$ depending on the EHEC strain [127]. Yuan and Yuk conducted a study investigating the impact of sublethal thymol exposure on virulence gene expression and virulence traits of *E. coli* O157:H7. Their findings indicated that bacteria exposed to sublethal concentrations of thymol during early stationary phase exhibited significantly reduced motility (which was reversible after stress removal), decreased biofilm formation, and diminished efflux pump activity. There was no observed induction of antibiotic resistance, and no significant alterations in adhesion and invasion capabilities on a human colon adenocarcinoma (Caco-2) cell line. qRT-PCR analysis demonstrated decreased expression of relevant virulence genes, including those associated with flagellar biosynthesis and function, biofilm formation regulation, multidrug efflux pumps, and components of the type III secretion system [85]. Similarly to carvacrol, thymol can effectively combat EHEC cells without triggering excessive production of toxins [143].

Essential oils pose a challenge in dispersing evenly in water due to their low solubility, leading to a heightened affinity for binding with food lipids and proteins. This diminished solubility reduces their antimicrobial effectiveness [95,150]. Researchers tested a nano-dispersed form encapsulated in whey protein isolate–maltodextrin conjugates against *E. coli* O157:H7 strains ATCC 43889 and 43894. The studies revealed that both nano-dispersed and free thymol exhibited an MIC of 300–500 ppm at pH 6.8 and a bacterial count reduction of 1.0 to 3.0 log CFU/mL after 48 h. At pH levels of 5.5 and 3.5, temperature variations did not significantly affect the antimicrobial activity of 500 mg/mL nano-dispersed thymol. The study highlighted the potent antimicrobial activity of transparent thymol nano-dispersions against various foodborne pathogens [126]. Another investigation explored nanoemulsions prepared using a mixture of ethyl lauroyl arginate (LAE) and lecithin. This combination resulted in stable translucent nanoemulsions of thymol with spherical droplets smaller than 100 nm, in contrast to the turbid emulsions formed using individual emulsifiers. Zeta-potential data suggested the formation of LAE–lecithin complexes, possibly through hydrophobic interaction. In 2% reduced-fat milk, nanoemulsions exhibited comparable antilisterial activities to free LAE in inhibiting *L. monocytogenes*. However, they were less effective against *E. coli* O157:H7 than free LAE, indicating a correlation with the availability of LAE, as observed in release kinetics. Therefore, while mixing LAE with lecithin improved the physical properties of EOC nanoemulsions, it did not enhance antimicrobial activities, especially against Gram-negative bacteria [95]. The nano form of essential oils provide a promising alternative with improved properties.

There are studies proving thymol's efficiency in food products. Pathogenic *E. coli* strains commonly encountered in meat and poultry include intestinal pathogenic *E. coli* (EHEC, but called iPEC in this study) and extraintestinal types such as uropathogenic *E. coli* (UPEC). In Chien et al.'s study, researchers compared the resistance of iPEC (O157:H7) to UPEC in chicken meat using high-pressure processing (HPP) both with and without thymol essential oil as a sensitizer, following the hurdle concept. UPEC exhibited slightly higher

resistance than *E. coli* O157:H7 at 450 and 500 MPa. A central composite experimental design was employed to assess the impact of pressure (300–400 MPa), thymol concentration (100–200 ppm), and pressure-holding time (10–20 min) on the inactivation of iPEC and UPEC strains in ground chicken. The hurdle approach aimed to reduce the intensity of high pressure and thymol levels applied to the food matrices, potentially minimizing damage to food quality post-treatment. The findings offer valuable insights into the survival of both O157:H7 and UPEC during HPP, whether in the presence or absence of thymol [151]. Osali et al. conducted a study to evaluate the antimicrobial properties of thymol (TH), an active essential oil, against *E. coli* O157:H7 in chicken tawook during storage at 4 and 10 °C. A marinade, comprising typical ingredients used in chicken tawook recipes, was prepared and combined with 1% and 2% v/v TH. This marinade, with or without essential oils (EOs), was applied to fresh chicken breast cubes inoculated with the foodborne pathogens. During storage at 10 °C, the marinade led to a reduction in *E. coli* O157:H7 levels on the chicken samples of approximately 3.3 log CFU/g on days 4 and 7 compared to untreated samples. At 4 °C, thymol contributed to a decrease in EHEC populations in chicken tawook of ≤ 2.4 log CFU/g compared to samples without it, where the reduction was ≤ 1.4 log CFU/g, regardless of storage duration. However, the addition of EOs did not significantly decrease *E. coli* O157:H7 populations in samples stored at 10 °C. Furthermore, increasing the concentration of EOs from 1% to 2% did not appear to have a significant impact on reducing the populations of the tested foodborne pathogens [152]. The study from 2021 examined the impact of a yogurt-based marinade combined with active essential oils—thymol, carvacrol, and cinnamaldehyde—on *E. coli* O157:H7 in camel meat stored at 4 and 10 °C. At 4 °C, marinating meat did not significantly alter bacterial counts, but at 10 °C, a notable decrease occurred on days 4 and 7 of storage, ranging from 2.7 to 2.1 CFU/g compared to untreated CM. Incorporating EOs enhanced the reduction in *E. coli* O157:H7, with a more pronounced effect observed when the EO concentration increased from 1% to 2% [153].

Thymol antimicrobial properties have led to its widespread use in the food industry as a natural preservative and in healthcare settings for its disinfectant properties. It is commonly found in mouthwashes, topical antiseptics, and disinfectant solutions. Overall, thymol represents a promising natural compound with potent antibacterial properties, and ongoing research continues to explore its therapeutic potential in various applications [141,148,149].

3.3.3. Menthol

Menthol is a naturally occurring compound found in the essential oil of mint plants, particularly peppermint (*Mentha piperita* L.) and spearmint (*M. spicata* L.). It is commonly used in various products such as cough drops, balms, and topical analgesics due to its cooling and soothing properties [154–156]. While menthol is primarily known for its sensory effects, there is limited evidence suggesting that it may possess certain antibacterial properties [154,157]. Some studies have explored the potential antimicrobial effects of menthol against various bacteria.

In a study from 2010, Landau and Shapira [129] discovered that preconditioning EHEC strains in escalating subinhibitory concentrations of menthol significantly enhances their resistance to menthol by 4- to 16-fold. Concurrently, observable morphological changes include the emergence of mucoid colonies and diminished biofilm production. Scanning electron microscopy (SEM) analysis reveals inhibited curli formation in cells adapted to menthol. In a laboratory *E. coli* strain, the expression of the *cpsB10* gene, responsible for colanic acid production, increases in response to SI menthol concentrations, while an *rcsC* mutant exhibits reduced expression, suggesting a partial involvement of the Rcs

phosphorelay system in mediating the menthol signal. Adaptation to menthol also leads to decreased expression of the locus of enterocyte effacement-encoded regulator (Ler). This decrease, coupled with reduced curli and biofilm formation and increased mucoidity, implies a general reduction in bacterial virulence after menthol adaptation. Consequently, these findings proposed menthol as a potential candidate in the emerging alternative approach of targeting bacterial virulence factors for the development of novel anti-infective agents [129]. Menthol MIC has been determined to be 1.0 and a minimum bactericidal concentration (MBC) to be 2.0 $\mu\text{g/mL}$ [119]. The antimicrobial activity of menthol is thought to be related to its ability to disrupt bacterial cell membranes and interfere with microbial growth. However, it is essential to note that the research on menthol antibacterial properties is not as extensive as that for other compounds, and more studies are needed to confirm and fully understand its mechanisms of action.

3.3.4. Linalool

Linalool is a naturally occurring terpene alcohol that belongs to the class of compounds known as monoterpenes. It is commonly found in the essential oils of various plants, including lavender, basil, rosewood, and coriander. While linalool is primarily recognized for its aromatic properties, there is some evidence to suggest that it may possess certain antibacterial properties [158]. Several studies have investigated the potential antimicrobial effects of linalool against various bacteria.

In their work, Soković et al. examined several essential oils and its main components. Linalool (27.2%) and linalyl acetate (27.5%) are the most abundant components in *Lavandula angustifolia* Mill. oil (yield is 3% (*v/w*)). Linalool is also the main component in *Ocimum basilicum* L. oil, with 69.3% (yield is 0.5% (*v/w*)) [119]. In the disc-diffusion method, the inhibition zone for linalool was 12.0 mm, and the MIC against *E. coli* O157:H7 was determined to be 6.0 and the MBC 7.0 $\mu\text{g/mL}$ [119]. The mechanism behind linalool's antibacterial properties is not fully understood, but it is believed to involve disruption of bacterial cell membranes, interference with membrane permeability, and inhibition of bacterial enzymes. Additionally, linalool may exert its antimicrobial effects by disrupting biofilm formation [158–160]. Notably, while there is promising research on the antibacterial properties of linalool, more studies are needed to better understand its efficacy, mechanisms of action, and potential applications in clinical settings. Additionally, the concentration of linalool and the specific bacterial strains being targeted can influence its effectiveness.

3.3.5. Limonene

Limonene, a cyclic monoterpene hydrocarbon, is commonly present in the peels of citrus fruits such as oranges, lemons, limes, and grapefruits, imparting their characteristic citrus aroma and flavor. Its versatile properties have led to widespread use across various industries, including food and beverage, cosmetics, cleaning products, and aromatherapy, owing to its pleasant fragrance and adaptable nature [161]. Although limonene is predominantly recognized for its aromatic attributes and its function as a solvent in cleaning solutions, it also demonstrates antibacterial properties. Studies indicate that limonene exhibits antimicrobial effects against diverse bacteria, encompassing both Gram-positive and Gram-negative strains. The mechanism involves the leakage of intracellular proteins, lipids, and nucleic acids, confirming membrane damage and disruption of cell permeability barriers. Additionally, the release of intracellular ATP further suggests disruption of the membrane barrier. Interaction with DNA underscores limonene's ability to unwind plasmids, potentially inhibiting DNA transcription and translation processes [161,162].

The MIC and MBC of limonene have been established to be 10 and 12 $\mu\text{g/mL}$, respectively. Limonene exhibited the least antibacterial efficacy compared to other compounds

tested, with an inhibition zone of 9 mm against EHEC bacteria [119]. Di Pasqua et al. (2006) determined alterations in membrane fatty acid composition induced by limonene. The concentration of unsaturated fatty acids (UFAs) notably increased when cells were exposed to limonene. This resulted from decreased palmitic acid levels and elevated concentrations of linoleaidic, docosanoic, and eicosapentaenoic acids [94]. Moreover, the anti-quorum-sensing potential of d-limonene in nano form against *E. coli* O157:H7 was investigated. The MIC was established at 5% (v/v), with sub-MIC levels of 2.5% (v/v) and 1.25% (v/v) selected for further evaluation. The study revealed that d-limonene nanoemulsion hindered *E. coli* biofilm formation by suppressing curli and extracellular polymeric substance production without impeding cell growth. Additionally, it reduced swimming and swarming abilities. Further analysis demonstrated interference with auto-inducer 2 (AI-2) communication by d-limonene nanoemulsion, which led to the repression of curli-related and AI-2 importer genes in *E. coli* [163]. Espina and Gelaw investigated the combined juice preservation techniques involving pulsed electric field (PEF) treatment and lethal heat supplemented with (+)-limonene against *E. coli* O157:H7. When applied in combination, the processes resulted in the inactivation of an additional 4.0 log or more compared to individual treatments. Consequently, this combination exhibited a synergistic effect on the overall inactivation. When (+)-limonene was applied separately at a concentration of 200 mL/L at 20 °C against an initial population of 3×10^7 CFU/mL of *E. coli* O157:H7 suspended in juices for 10 min, less than 0.5 log of the initial population were inactivated. Conversely, a separate PEF treatment in the absence of (+)-limonene resulted in the inactivation of less than 0.5 log of the initial *E. coli* O157:H7 population and caused sub-lethal injury to the bacterial outer membrane in less than 1.0 log of surviving cells. The combined process involving PEF with (+)-limonene resulted in additive final inactivation levels, equivalent to the sum of the inactivation levels of both treatments applied separately. No additional inactivation was observed due to the simultaneous application of lethal heat treatment in the presence of (+)-limonene [164].

It is important to note that limonene efficacy can vary depending on factors such as concentration, bacterial strain, and environmental conditions. Further research is needed to fully understand the antimicrobial properties of limonene and its potential applications in clinical settings. Overall, limonene represents a natural compound with diverse biological activities, and ongoing studies continue to explore its therapeutic potential in various contexts, including antimicrobial and antibacterial applications.

3.3.6. Other Terpenes and Terpenoids

Several other natural compounds exhibit significant antimicrobial properties against EHEC. Compounds such as terpineol, camphor, and pinene primarily disrupt bacterial cell membranes and interfere with metabolic functions, leading to bacterial eradication [133,165,166].

Terpineol, a monoterpene alcohol found in pine oil, cajuput oil, and eucalyptus oil, has a hydroxyl group that interacts with bacterial carbohydrates and other chemical groups [133,167]. This interaction leads to changes in phospholipid polar head groups and decreased membrane fluidity, disrupting the electron transport chain and causing a collapse of membrane potential and pH balance. At a concentration of 0.8%, α -terpineol reduces *E. coli* O157 populations by about 5.6 log CFU/mL within just one hour, attributed to ATP leakage and membrane disruption [133]. Similarly, camphor, extracted from the wood of the camphor tree (*Cinnamomum camphora* Ness et Eberm.) or synthesized from turpentine oil, disrupts bacterial cell membranes and interferes with metabolic processes [165,166]. It demonstrates significant antibacterial activity against the O157:H7 strain, with an MIC of 7.0 μ g/mL and MBC of 8.0 μ g/mL. A zone of growth inhibition measuring 13 mm was

observed [119], indicating its effectiveness in hindering bacterial proliferation. However, not all studies have found camphor to be highly effective; for instance, when combined with high hydrostatic pressure (HHP), camphor achieved less than a 2.0 log reduction in bacterial counts, suggesting variable effectiveness depending on environmental conditions [130]. Pinene, a bicyclic monoterpene hydrocarbon found predominantly in coniferous trees, exists in two isomeric forms: α -pinene and β -pinene [168]. Both isomers inhibit microbial cell membrane integrity, exhibiting inhibition zones of 10 mm against the *E. coli* strain tested. They have similar MIC values of 8.0 $\mu\text{g/mL}$ and MBCs of 10.0 $\mu\text{g/mL}$ [119]. Despite their antibacterial properties, pinene isomers do not exhibit significant antioxidative activity [131].

In contrast, compounds like geraniol and isolimonic acid focus on inhibiting biofilm formation and bacterial adhesion, which are crucial for bacterial colonization and infection. Geraniol, found in the essential oils of roses, citronella, and geraniums [169], acts as an uncompetitive inhibitor of glucosyltransferase, an enzyme essential for biofilm formation. It reduces bacterial adhesion on stainless steel surfaces and decreases glucan production, with an MIC of 3.0 mg/mL against *E. coli* O157:H7. Docking analyses suggest that geraniol interacts with the helix finger of glucosyltransferase responsible for polymer production, thereby inhibiting biofilm formation [136]. Isolimonic acid, a triterpenoid compound isolated from certain pine trees, is a strong inhibitor of biofilm formation and attachment in EHEC [138,139,170]. It acts by suppressing the expression of LEE and flagellar operons, disrupting the type III secretion system. Isolimonic acid shows significant inhibition of biofilm formation, with an inhibitory concentration (IC₂₅) of 19.7 μM , and has been shown to inhibit the formation and attachment of biofilms to Caco-2 cells. It is believed to exert its effects by inhibiting AI-3/epinephrine-mediated cell–cell signaling through the QseBC and QseA pathways, although the precise mechanisms remain to be fully elucidated [138].

Eucalyptol (1,8-cineole), commonly extracted from eucalyptus species like *Eucalyptus globulus* Labill. and *Ecklonia radiata* J. Agardh, as well as other aromatic plants like rosemary and tea tree, enhances its antimicrobial efficacy through high water solubility, facilitating diffusion into agar media and interaction with microbial cells [119,171,172]. It elongates the lag phase of bacterial growth, inhibiting proliferation over time [131]. Eucalyptol exhibits an inhibition zone of 18.0 mm against *E. coli* O157, with bacteriostatic activity observed at concentrations as low as 6.0 $\mu\text{g/mL}$ (MIC) and bactericidal activity at 8.0 $\mu\text{g/mL}$ (MBC) [119]. At a concentration of 0.6%, it has been found to elongate the lag phase of *E. coli* O157:H7, indicating its potential to inhibit bacterial growth over time. However, it shows weak antioxidant activity by the ferric reducing antioxidant power (FRAP) method and almost no free radical scavenging activity with the DPPH method [131].

These compounds, through their shared and unique mechanisms of action at specific concentrations, demonstrate the potential for developing effective natural antimicrobial agents. Oxygenated monoterpenes—eucalyptol, terpineol, geraniol, and camphor—generally exhibit higher antimicrobial potential compared to hydrocarbon monoterpenes like pinene. This increased efficacy is partly due to their higher water solubility, which facilitates diffusion through agar media and interaction with microbial cells [119]. Functional groups such as hydroxyl groups in terpineol and geraniol enhance their ability to form hydrogen bonds with microbial cell components, disrupting membrane integrity and metabolic processes [119,133,167]. These findings underscore the potential of natural compounds in developing new antimicrobial agents or natural preservatives for the food and pharmaceutical industries. The increasing consumer preference for natural alternatives to chemical bactericides, coupled with concerns about microbial resistance, positions these compounds as promising candidates for enhancing food safety and developing novel therapeutic applications. However, further research is necessary to fully

understand their mechanisms of action, optimize their efficacy, and ensure their safety in practical applications.

4. Perspectives

With the rise of antibiotic resistance, plant-derived compounds—or phytochemicals—are increasingly recognized as promising alternatives and adjuncts for combating bacterial infections. Compounds like polyphenols, terpenes, and ITCs exhibit diverse antimicrobial mechanisms, including membrane disruption, inhibition of virulence signaling pathways, and suppression of genes crucial for bacterial pathogenicity (Figure 5). While certain phytochemicals, such as thymol and eucalyptol, are already FDA-approved for over-the-counter antiseptic products, others, like EGCg and sulforaphane, are under active investigation in clinical trials. Nevertheless, phytochemicals offer significant potential for integration into existing food-safety protocols as natural antimicrobials [173–175]. These compounds can serve as secondary hurdles, complementing established practices like pasteurization, refrigeration, and chemical preservatives, to provide additional protection against microbial contamination. Effective integration requires careful consideration of several factors, including their antimicrobial efficacy under typical storage and processing conditions, and their compatibility with various food matrices. Furthermore, compliance with regulatory frameworks is necessary, including safety evaluations, toxicological assessments, and adherence to labeling standards set by regulatory agencies such as the Food and Drugs Administration (FDA) and the European Food Safety Authority (EFSA). This review underscores the potential of these bioactive compounds to modulate EHEC virulence specifically and highlights how their unique mechanisms can effectively support or potentially replace traditional antibiotics. As research progresses, the distinct properties of phytochemicals may offer safer and more targeted solutions for controlling bacterial pathogens without fostering resistance.

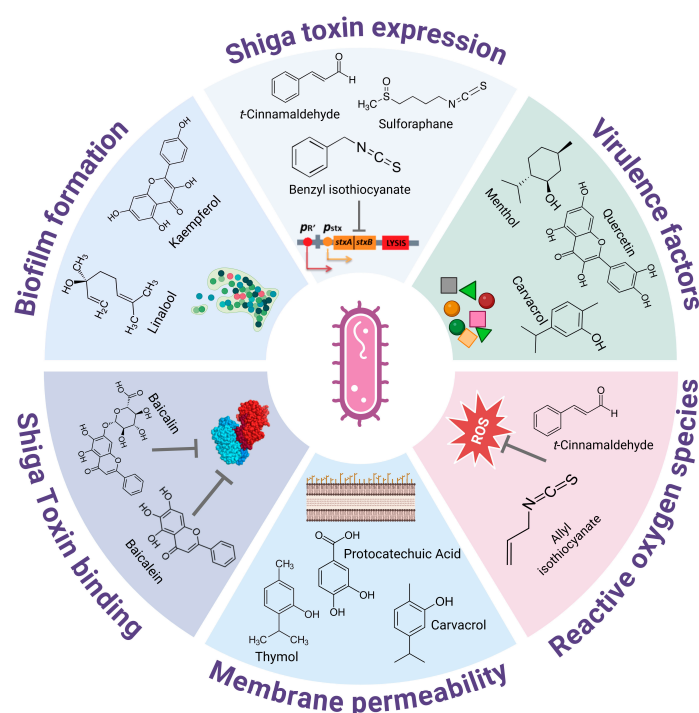


Figure 5. Representation of natural compounds and their targeted actions against enterohemorrhagic *E. coli* (EHEC) pathogenesis. The highlighted pathways show the compounds' potential to reduce bacterial virulence, toxin production, and ROS generation, while enhancing membrane disruption and biofilm inhibition. Created in BioRender. <https://BioRender.com/q50t463>.

Challenges and Future Research Directions

For phytochemicals to move effectively from bench to bedside, several challenges must be addressed:

- **Integrating phytochemicals into existing food safety protocols or regulatory frameworks:** Effective incorporation of phytochemicals requires establishing their role in promoting functional foods or “superfoods”, and standardizing food products for consistent phytochemical quality, content, and aligning dietary recommendations. Phytochemicals can serve as alternative strategies to antibiotic treatments, supporting a holistic One Health approach that addresses human, animal, and environmental health while minimizing chemical use.
- **Bioavailability and Targeted Delivery:** Ensuring that phytochemicals maintain stability and bioactivity in the gastrointestinal environment is critical for controlling EHEC effectively. Advanced delivery systems, such as encapsulation or nanoformulations, could enhance bioavailability and direct these compounds to targeted sites within the gut.
- **Clinical Trials for EHEC-Specific Applications:** While preclinical data are promising, rigorous clinical studies focused explicitly on EHEC are essential to confirm the safety, efficacy, and optimal dosages of these compounds in human populations. Current evidence from applications beyond EHEC provides a strong foundation, but EHEC-specific trials will be necessary to substantiate therapeutic potential.
- **Interactions with the Gut Microbiome:** Understanding how phytochemicals influence the host microbiome is crucial to avoid unintended disruption of beneficial gut flora. Studying these interactions can help ensure that treatment selectively targets EHEC while maintaining the balance of the broader microbial community.
- **Interactions with Other Therapeutic Agents:** Phytochemicals may enhance or interfere with conventional antibiotics and other drugs. Research should investigate these interactions to optimize combinations, aiming to boost efficacy and reduce potential conflicts in treatment.
- **Green-Synthesized Materials:** Secondary plant metabolites are utilized as biological precursors and active components of new nanomaterials such as metal oxides, delivery systems, bio-sensors, or antiseptic fabrics.

5. Conclusions

This review contributes to the ongoing search for innovative strategies against EHEC, especially given the limitations of antibiotics for treating this infection. Phytochemical-based therapies, grounded in a targeting of EHEC virulence mechanisms, offer an alternative to traditional antibiotics and may lead to more sustainable, less resistance-prone treatments. Moving forward, the development of these plant-derived agents as therapeutic interventions should be prioritized, not only for EHEC but potentially also for a broader range of bacterial infections where antibiotic resistance is a concern.

Author Contributions: Conceptualization, D.N.; validation, D.N. and A.S.-P.; investigation, P.S., M.K., A.S.-P. and D.N.; writing—original draft preparation, D.N.; writing—review and editing, P.S., M.K., A.S.-P. and D.N.; visualization, P.S.; supervision, D.N. and A.S.-P.; project administration, D.N.; funding acquisition, D.N. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Science Center SONATA, grant no. UMO-2018/31/D/NZ7/02258 (D.N.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We sincerely acknowledge the colleagues from the Department of Bacterial Molecular Genetics, University of Gdansk, for their support in inspiring debates on this topic. We thank the reviewers for their comments and suggestions, which helped improve the manuscript.

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

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12. OŚWIADCZENIA WSPÓŁAUTORÓW

PUBLIKACJA 1. „*Antibacterial activity of t-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic Escherichia coli (EHEC)* „

Gdańsk, 12.06.2026r.

mgr Monika Karczewska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Antibacterial activity of t-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)” obejmował planowanie i wykonywanie badań eksperymentalnych, analizę wyników, analizę transkryptomiczną oraz przygotowanie manuskryptu.



Gdańsk, 12.06.2026r.

mgr Ai Yan Wang

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Antibacterial activity of *t*-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)” obejmował udział w realizacji badań eksperymentalnych oraz analizie wyników.

A handwritten signature in black ink, appearing to read 'Wang A. Yan', written in a cursive style.

Gdańsk, 12.06.2026r.

dr hab. Magdalena Narajczyk
Laboratorium Bioobrazowania
Wydział Biologii UG

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Antibacterial activity of *t*-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)” obejmował wykonanie i analizę badań przeprowadzonych z wykorzystaniem technik mikroskopii elektronowej.

Magdalena Narajczyk

Dr hab. med. Bartosz Słomiński
Katedra i Zakład Immunologii Medycznej
Wydział Lekarski
Gdański Uniwersytet Medyczny
ul. Dębinki 1
80-211 Gdańsk

Gdańsk, 12.06.2026

Oświadczenie

Oświadczam, że mój wkład w powstanie pracy pt. „Antibacterial activity of t-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic Escherichia coli (EHEC)” obejmował udział w analizie wyników oraz konsultacje merytoryczne.

GDĄSKI UNIERSYTET MEDYCZNY
Katedra i Zakład Immunologii
Medycznej
12.06.2026 
.....
dr hab. med. Bartosz Słomiński
(data, podpis)

Gdańsk, 12.06.2026r.

prof. dr hab. Agnieszka Szalewska-Pałasz

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Antibacterial activity of t-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)” obejmował współtworzenie koncepcji badań, nadzór merytoryczny oraz redakcję manuskryptu.

A. Szalewska-Pałasz

Gdańsk, 12.06.2026r.

dr Dariusz Nowicki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Antibacterial activity of *t*-cinnamaldehyde: An approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)” obejmował opracowanie koncepcji badań, nadzór nad realizacją projektu, analizę wyników oraz przygotowanie i redakcję manuskryptu.



22 06 2026

PUBLIKACJA 2. „*Increased Levels of (p)ppGpp Correlate with Virulence and Biofilm Formation, but Not with Growth, in Strains of Uropathogenic Escherichia coli*”

Gdańsk, 12.06.2026r.

mgr Monika Karczewska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Increased levels of (p)ppGpp correlate with virulence and biofilm formation, but not with growth, in strains of uropathogenic *Escherichia coli*” obejmował planowanie badań, wykonywanie eksperymentów, analizę wyników oraz przygotowanie manuskryptu.



Gdańsk, 12.06.2026r.

mgr Patryk Strzelecki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Increased levels of (p)ppGpp correlate with virulence and biofilm formation, but not with growth, in strains of uropathogenic *Escherichia coli*” obejmował udział w realizacji eksperymentów oraz analizie danych.

Strzelecki Patryk

Gdańsk, 12.06.2026r.

mgr Krystyna Bogucka

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Increased levels of (p)ppGpp correlate with virulence and biofilm formation, but not with growth, in strains of uropathogenic *Escherichia coli*” obejmował udział w pracach mikrobiologicznych.

Krystyna Bogucka

Gdańsk, 12.06.2026r.

prof. dr hab. Katarzyna Potrykus

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy: Karczewska, M.; Strzelecki, P.; Bogucka, K.; Potrykus, K.; Szalewska-Pałasz, A.; Nowicki, D. Increased Levels of (p)ppGpp Correlate with Virulence and Biofilm Formation, but Not with Growth, in Strains of Uropathogenic *Escherichia coli*. *Int. J. Mol. Sci.* **2023**, *24*, 3315. <https://doi.org/10.3390/ijms24043315>, obejmował interpretację wyników oraz korektę manuskryptu.

 KIEROWNIK
Katedra Genetyki Molekularnej
Bakterii
K. Potrykus
prof. dr hab. Katarzyna Potrykus

Gdańsk, 12.06.2026r.

prof. dr hab. Agnieszka Szalewska-Pałasz

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Increased levels of (p)ppGpp correlate with virulence and biofilm formation, but not with growth, in strains of uropathogenic *Escherichia coli* „ obejmował współtworzenie koncepcji badań, nadzór merytoryczny oraz redakcję pracy.

A. Szalewska-Pałasz

Gdańsk, 12.06.2026r.

dr Dariusz Nowicki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Increased levels of (p)ppGpp correlate with virulence and biofilm formation, but not with growth, in strains of uropathogenic *Escherichia coli*” obejmował opracowanie koncepcji badań, nadzór nad realizacją projektu oraz przygotowanie i redakcję manuskryptu.



22 06 2026

PUBLIKACJA 3. „*Trans-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic Escherichia coli*”

Gdańsk, 12.06.2026r.

mgr Monika Karczevska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „*Trans-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic Escherichia coli*” obejmował planowanie i realizację badań eksperymentalnych, analizę wyników, analizę transkryptomiczną oraz przygotowanie manuskryptu.

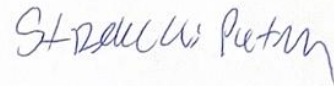


Gdańsk, 12.06.2026r.

mgr Patryk Strzelecki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „*Trans*-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic *Escherichia coli*” obejmował udział w realizacji badań eksperymentalnych oraz analizie danych.



Gdańsk, 12.06.2026r.

dr inż. Agnieszka Pyrczak-Felczykowska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „*Trans*-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic *Escherichia coli*” obejmował wykonanie badań cytotoksyczności oraz interpretację uzyskanych wyników.



Gdańsk, 12.06.2026r.

dr Katarzyna Zima

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „*Trans*-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic *Escherichia coli*” obejmował udział w realizacji badań z wykorzystaniem komórek eukariotycznych oraz analizie wyników.



Gdańsk, 12.06.2026r.

prof. dr hab. Agnieszka Szalewska-Pałasz

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „*Trans*-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic *Escherichia coli*” obejmował współtworzenie koncepcji badań, nadzór merytoryczny oraz redakcję manuskryptu.



Gdańsk, 12.06.2026r.

dr Dariusz Nowicki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „*Trans-cinnamaldehyde triggers stringent response-mediated virulence attenuation in pathogenic Escherichia coli*” obejmował opracowanie koncepcji badań, nadzór nad realizacją projektu, analizę wyników oraz przygotowanie i redakcję manuskryptu.



22 06 2026

PUBLIKACJA 4. (nierecenzowany manuskrypt) „*Spicing up urinary tract infections: synergistic action of fosfomycin with trans-cinnamaldehyde - insight to the mode of action*”

Gdańsk, 22.06.2026r.

mgr Monika Karczevska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie manuskryptu pt. „*Spicing up urinary tract infections: synergistic action of fosfomycin with trans-cinnamaldehyde – insight to the mode of action*” obejmował współtworzenie koncepcji badań, opracowanie metodologii, prowadzenie badań eksperymentalnych, gromadzenie i analizę danych, przygotowanie materiałów graficznych oraz opracowanie pierwszej wersji manuskryptu.



Gdańsk, 22.06.2026r.

mgr Patryk Strzelecki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie manuskryptu pt. „Spicing up urinary tract infections: synergistic action of fosfomycin with *trans*-cinnamaldehyde – insight to the mode of action” obejmował udział w realizacji badań eksperymentalnych oraz opracowaniu uzyskanych wyników.

Strzelecki

Gdańsk, 22.06.2026r.

dr Monika Maciąg-Dorszyńska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie manuskryptu pt. „Spicing up urinary tract infections: synergistic action of fosfomicin with *trans*-cinnamaldehyde – insight to the mode of action” obejmował udział w realizacji badań eksperymentalnych, opracowaniu metodologii oraz gromadzeniu i analizie danych.



Gdańsk, 22.06.2026r.

dr Małgorzata Kapusta

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie manuskryptu pt. „Spicing up urinary tract infections: synergistic action of fosfomicin with *trans*-cinnamaldehyde – insight to the mode of action” obejmował udział w opracowaniu metodologii oraz wykonaniu i analizie badań z wykorzystaniem technik mikroskopowych.

Małgorzata Kapusta

Gdańsk, 22.06.2026r.

dr inż. Agnieszka Pyrczak-Felczykowska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie manuskryptu pt. „*Spicing up urinary tract infections: synergistic action of fosfomycin with trans-cinnamaldehyde – insight to the mode of action*” obejmował udział w realizacji badań eksperymentalnych oraz opracowaniu metodologii badań.



Gdańsk, 22.06.2026r.

prof. dr hab. Agnieszka Szalewska-Pałasz

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie manuskryptu pt. „Spicing up urinary tract infections: synergistic action of fosfomycin with *trans*-cinnamaldehyde – insight to the mode of action” obejmował udział w walidacji wyników, nadzorze merytorycznym nad realizacją projektu oraz recenzji i redakcji manuskryptu.

A. Szalewska-Pałasz

Gdańsk, 22.06.2026r.

dr Dariusz Nowicki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie manuskryptu pt. „Spicing up urinary tract infections: synergistic action of fosfomicin with *trans*-cinnamaldehyde – insight to the mode of action” obejmował współtworzenie koncepcji badań, udział w realizacji badań eksperymentalnych, opracowanie metodologii, walidację wyników, analizę i wizualizację danych, pozyskanie finansowania, nadzór nad projektem oraz przygotowanie i redakcję manuskryptu.



PUBLIKACJA 5. ” *Phytochemicals Controlling Enterohemorrhagic Escherichia coli (EHEC) Virulence—Current Knowledge of Their Mechanisms of Action* ”

Gdańsk, 22.06.2026r.

mgr Patryk Strzelecki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie pracy pt. „Phytochemicals Controlling Enterohemorrhagic *Escherichia coli* (EHEC) Virulence—Current Knowledge of Their Mechanisms of Action” obejmował współtworzenie przeglądu literaturowego oraz synteze wniosków, redakcji manuskryptu i wizualizacji.

Strzelecki

Gdańsk, 22.06.2026r.

mgr Monika Karczewska

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie publikacji pt. „**Phytochemicals Controlling Enterohemorrhagic *Escherichia coli* (EHEC) Virulence—Current Knowledge of Their Mechanisms of Action**” obejmował współtworzenie przeglądu literaturowego oraz synteze wniosków, redakcji manuskryptu



Gdańsk, 22.06.2026r.

prof. dr hab. Agnieszka Szalewska-Pałasz

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie publikacji pt. „**Phytochemicals Controlling Enterohemorrhagic *Escherichia coli* (EHEC) Virulence—Current Knowledge of Their Mechanisms of Action**” obejmował współtworzenie przeglądu literaturowego walidacji danych oraz syntezie wniosków, redakcji manuskryptu, współnadzorce nad projektem.



Gdańsk, 22.06.2026r.

dr Dariusz Nowicki

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie publikacji pt. „**Phytochemicals Controlling Enterohemorrhagic *Escherichia coli* (EHEC) Virulence—Current Knowledge of Their Mechanisms of Action**” obejmował konceptualizacji przeglądu literaturowego, analizie danych oraz ich walidacji, redakcji manuskryptu i nadzorze nad prowadzonymi badaniami.



13. POZOSTAŁA AKTYWNOŚĆ NAUKOWA

Artykuły naukowe niewchodzące w skład rozprawy:

1. Dietary isothiocyanates, sulforaphane and 2-phenethyl isothiocyanate, effectively impair *Vibrio cholerae* virulence

Krause Klaudyna, Pyrczak-Felczykowska Agnieszka, Karczewska Monika [i in.], International Journal of Molecular Sciences, (2021), vol. 22, nr 19, s.1-19, Numer artykułu:10187

DOI:10.3390/ijms221910187

2. Evaluation of the anti-shigellosis activity of dietary isothiocyanates in *Galleria mellonella* larvae

Nowicki Dariusz, Krause Klaudyna, Karczewska Monika [i in.], Nutrients, (2021), vol. 13, nr 11, s.1-12, Numer artykułu:3967. DOI:10.3390/nu13113967

3. How to tackle bacteriophages: the review of approaches with mechanistic insight

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4. Toxin-antitoxin genes are differentially expressed in *Escherichia coli relA* and *spoT* mutants cultured under nitrogen, fatty acid, or carbon starvation conditions

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Doniesienia konferencyjne o tematyce rozprawy:

1. The biofilm formation and the role of (p)ppGpp in the uropathogenic *Escherichia coli* strains

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2. Antibacterial activity of *t*-cinnamaldehyde: an approach to its mechanistic principle towards enterohemorrhagic *Escherichia coli* (EHEC)

Karczewska Monika, Wang Ai Yan, Martyn Sylwia, Nowicka Wioletta, Szalewska-Pałasz Agnieszka, Nowicki Dariusz, (2022), 5th International Caparica Congress in Antibiotic Resistance, poster

3. *Escherichia coli* and the role of (p)ppGpp in the biofilm formation

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4. *Trans* cinnamaldehyde effectively impairs EHEC virulence through *relA* dependent induction of stringent response

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Inne osiągnięcia naukowe i badawcze:

1. **Laureatka programu małych grantów UGrants-START** realizowanego na Uniwersytecie Gdańskim nr 533-BG10-GS11-25, (2025–2026)

2. Współautor zgłoszeń patentowych:

- Monika Karczewska, Agnieszka Szalewska-Pałasz, Dariusz Nowicki. *Kompozycja farmaceutyczna o działaniu przeciwbakteryjnym wobec *Escherichia coli*, sposób jej wytwarzania oraz zastosowanie*. Zgłoszenie patentowe nr. 1457A - P.455870 z dnia 27.05.2026 r. Urząd Patentowy Rzeczypospolitej Polskiej.
- Monika Karczewska, Agnieszka Szalewska-Pałasz, Dariusz Nowicki. *Kompozycja farmaceutyczna o działaniu przeciwbakteryjnym wobec *S. aureus*, sposób jej wytwarzania oraz zastosowanie*. Zgłoszenie patentowe nr. 1940A - P.455871 z dnia 27.05.2026 r. Urząd Patentowy Rzeczypospolitej Polskiej.