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Empowering colistin effectiveness: *Origanum majorana* essential oil enhances antibacterial and antibiofilm potency against *mcr-1*-positive Gram-negative bacteria, preventing drug resistance development

Zine El Abidine Bzazou El Ouazzani[1]; Laila Reklaoui[1]; Houda Benaicha[1,2]; Said Barrijal[1*]

[1]Laboratory of Biotechnological Valorization of Microorganisms, Genomics and Bioinformatics, Department of Life Science, Abdelmalek Essaadi University, Tangier, Morocco; [2]Institut Supérieur des Professions Infirmières et Techniques de Santé de Tanger, Tangier, Morocco

[*]Corresponding author E-mail: barrijal@yahoo.fr

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Abstract

Escherichia coli and *Klebsiella pneumonia*; along with other Gram-negative bacteria (GNB), are known for their resilience to a broad spectrum of antibiotics, including last-resort options such as colistin, which is regarded as the last line of defense in treating multidrug-resistant (MDR) infections. This elevated level of resistance presents a substantial threat to the healthcare system, paving the way for a post-antibiotic era. In the present study, a checkerboard assay was employed to pinpoint the optimal combinations of *Origanum majorana* (OM) essential oil and colistin (CS) for the purpose of enhancing colistin's antibacterial and antibiofilm activities (FICI ≤ 0.5). The investigation focused on eight colistin-resistant (CS_R) bacterial isolates, with a particular focus on two strains carrying the mobile colistin resistance *mcr-1* gene. The CS@OM combination resulted in a 2- to 16-fold improvement of colistin efficacy, resulting in a biofilm eradication rate ranging from ~ 52 % to 82 %. Furthermore, it evinced a complete eradication of the free-floating cells of the isolates, inclusive of those harboring the *mcr-1* gene. The bactericidal activity of the CS@OM combination was achieved through various mechanisms, including bacterial membrane disruption and leakage of internal bacterial constituents, leading to bacterial death. Consequently, the CS@OM combination evinced a high capacity to prevent the development of drug resistance in the examined isolates. To the best of our knowledge, this is the first study to appraise the combination of colistin and *Origanum majorana* essential oil against colistin-resistant isolates, including those carrying *mcr-1* gene with the aim of regaining colistin susceptibility.

Keywords: Colistin resistance, *Origanum majorana*, *mcr-1* gene, *E. coli*, *K. pneumonia*

1. Introduction

Escherichia coli (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are among other Gram-negative bacteria (GNB) that have emerged as a significant public health concern due to the rise of antibiotic-resistant strains (Thando *et al*., 2023; Sina *et al*., 2024). GNB display resistance to a wide array of antibiotics, including last-resort options such as carbapenem, tigecycline, and polymyxins (Brink, 2019; Elnasser *et al*., 2021). While *E. coli* typically inhabits harmlessly the human intestinal tract, it has the potential to evolve into a pathogenic form, giving rise to various diseases, including septicemia, meningitis, diarrhea, hemolytic uremic syndrome, and urinary tract infections (UTIs). This transition occurs upon the acquisition of virulence factors by the bacterium or through person-to-person transmission (Pokharel *et al*., 2023). Uropathogenic (UPEC) *E. coli* accounts for approximately 80 % of community-acquired and 30 % of nosocomial-acquired UTIs (Makvana and Krilov, 2015). *K. pneumoniae*, on the other hand, is an opportunistic pathogen affecting the urinary tract of immunocompromised and hospitalized patients and is frequently associated with catheter-associated UTIs. Managing infections of this nature poses a significant challenge, given that the majority of clinical isolates demonstrate resistance to multiple antibiotics. This resistance is attributed to the bacterial capacity to form biofilms and the restricted permeability of their cell walls (Hazel *et al*., 2024; Swedan and Aldakhily, 2024).

In response to the scarcity of therapeutic options available for treating GNB infections, healthcare professionals are currently reassessing the use of previously abandoned antibiotics due to their toxicity. Colistin (CS), for instance, was extensively abandoned in the period between the 1980s and 2000s due to its detrimental impact on the kidney and nervous system cells. During this period, aminoglycosides emerged as a favored alternative, characterized by lower toxicity and enhanced efficacy (Biswas *et al*., 2012, El-Khatib and Basyony, (2024). However, with the emergence of carbapenem-producing *Enterobacteriaceae* (CPE), colistin has become the ultimate option for treating such type of infections, and its usage has consequently increased over the past decade (Van and Doi, 2017). Nevertheless, the extensive use of colistin in humans and animals has led to the emergence of GNB strains that are resistant to this antibiotic (Dortet *et al*., 2016). The escalating prevalence of bacterial drug resistance and the shortage of newly developed antibiotics have prompted experts to explore alternative approaches to enhance the effectiveness of antibiotics (Murugaiyan *et al*., 2022). One such approach involves combining antibiotics with natural compounds, specifically essential oils (EOs) (Kon and Rai, 2013; Aziz *et al*., 2024). Essential oils are made up of volatile aromatic chemical compounds, consisting of major and minor components that vary depending on the type of EO (Sadgrove *et al*., 2022). Noteworthy research has underscored the efficacy of combining antibiotics with EOs, including *Tanacetum vulgare*/ cephalosporins, carbapenems, and *Mentha arvensis*/ chloramphenicol, ampicillin, and erythromycin, as a potent strategy for enhancing the antibacterial activity of antibiotics (Roman *et al*., 2023; Sharma *et al*., 2023). However, no studies have yet been conducted to investigate the potential benefits of combining colistin with *O. majorana* (OM) EO. The objective of this study was to establish the optimal combination of colistin and OM EO for effectively addressing CS_R *E. coli* and *K. pneumoniae* isolates. Assessment of these combinations encompasses their antibacterial activity and their capacity to eradicate the bacterial isolate's preformed biofilms. Additionally, the CS@OM combination has also exhibited a notable ability to impede the development of drug resistance in the tested bacteria.

2. Materials and methods

2.1. Study design

For microbial analysis, eight CS_R *Enterobacteriaceae* clinical isolates were selected, consisting of four *E. coli* and four *K. pneumonia* isolates. Selection of isolates was derived from an ongoing study conducted in our laboratory based on their resistance to colistin (Unpublished data). Before proceeding with the analysis, the isolates were characterized based on their antibiotic resistance profile and presence of the *mcr-1* gene using polymerase chain reaction (PCR) technique (Table 1). *Origanum majorana* plant was collected from Tetouan city region in North Morocco, and its essential oil was obtained by solid-liquid extractions using a Soxhlet extractor (Humeau laboratory, France). The synergistic effects of CS@OM were investigated using various assays such as checkerboard, time-kill curves, biofilm eradication assays, and bacterial intracellular components leakage (β-galactosidase enzyme, DNA/RNA, and proteins) through the bacterial cell wall, and ability of the combination to escaping drug development.

Table 1: Antibiotic resistance profile of the eight tested bacterial isolates

Isolate code	Bacterial species	*mcr*-1 gene	Antibiotics resistance profile
TT-7	*E. coli*	+	NA, CPR, AMX, TOB, CS
N67	*K. pneumoniae*	-	AMX, CS
O48	*K. pneumoniae*	+	AMX, CEF, CS
TT-18	*E. coli*	-	AMX, CPR, CEF, NA, CS
TT-25	*K. pneumoniae*	-	CPR, CXT, CEF, CAZ, NA, TOB, CS
TT-74	*E. coli*	-	NA, CPR, AMX, CS
TT-82	*K. pneumoniae*	-	AMX, CIP, CEF, NA, CS
O83	*E. coli*	-	AMX, CAZ, AMK, TOB, CEF, NA, IMP, CS

Where; AMX: Amoxicillin, CEF: Cephalotin, CXT: Cefotaxime, CAZ: Ceftazidime, IMP: Imipenem, TOB: Tobramycin, AMK: Amikacin, NA: Nalidixic Acid, CPR: Ciprofloxacin, CS: Colistin

2.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of colistin and *Origanum majorana*

The antibacterial activity of OM EO and CS against the eight bacterial isolates was assessed using the broth microdilution method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI. 2020). The bacterial cultures were incubated at 37 °C overnight in Mueller-Hinton II cation-adjusted broth (Sigma-Aldrich, France). Subsequently, the developing cultures were diluted to an optical density OD 600 nm (OD_{600}) = 0.01 [~10^5-10^6 colony forming units (cfu/ ml)]. Afterward, 50 µl of the diluted bacterial cultures were mixed with 50 µl of OM (diluted 1:1 in dimethyl sulfoxide) at concentrations ranging from 0.97 to 31.2 to µg/ ml, and 50 µl of colistin at concentrations ranging from 0.5 to 16 to µg/ ml in 96-well plates. The mixture was then incubated at 37 °C for 24 h with

continuous shaking at 150 rpm. After incubation, bacterial growth was measured (OD_{600}) using a microplate reader (AccuSkan™, Fisher Scientific, France). The lowest concentrations of OM or CS without turbidity (OD_{600} ~ 0) were considered as MICs (Morena *et al*., 2022). Additionally, 10 µl of the samples were transferred to Coliform ChromoSelect agar (Sigma-Aldrich, France) plates and incubated for 24 h at 37 °C. The developing colonies were counted to determine the MBC (Morena *et al*., 2022). The results are reported as mean values ± SD (n = 3).

2.3. Two-dimensional checkerboard assay

The potential synergistic interactions between CS and OM were assessed using a checkerboard assay (Wickremasinghe *et al*., 2021). In a 96-well plate, 50 µl of OM underwent serial 2-fold dilution along the X-axis, while 50 µl of CS underwent similar dilution along the Y-axis in Mueller-Hinton II cation-adjusted broth. This resulted in a 6 × 7 matrix consisting of different concentrations of both agents in each well. Subsequently, each well was inoculated with 50 µl of an overnight bacterial culture, with a concentration of ~ 10^5-10^6 cfu/ ml, followed by incubation at 37 °C for 24 h with shaking at 150 rpm. Post-incubation, 10 µl of 0.015 % (w/v) resazurin sodium salt (Sigma-Aldrich, France) was added to each well, and a 30-min incubation period was ensued to facilitate visualization of bacterial growth via a color change from purple to pink, indicative of bacterial growth (Martínez-Servat *et al*., 2018). The results are reported as mean values ± SD (n = 3).

The Fractional Inhibitory Concentration Index (FICI) was calculated to evaluate the synergetic effect of the combination, using the following formula reported by Wickremasinghe *et al*., (2021):

$$\Sigma\ FICI = [FIC\ (A) = \frac{MIC\ (A)\ in\ combination}{MIC(A)\ alone}] + [FIC(B) = \frac{MIC\ (B)\ in\ combination}{MIC(B)\ alone}]$$

Where; FICI ≤ 0.5: synergistic effect; 0.5 < FICI ≤ 1: additive effect; 1 < FICI ≤ 4: indifferent; and FICI > 4: antagonistic effect

2.4. Biofilm eradication

The effect of CS@OM combination on the pre-existing bacterial biofilm was assessed using crystal violet (CV) assay (Ivanova *et al*., 2020). Briefly, an overnight culture of each isolate was diluted in Mueller-Hinton II cation-adjusted broth (OD_{600} = 0.01). Subsequently, 100 µl of these cultures were added individually to a 96-well microplate, and the biofilm was allowed to form for 24 h at 37 °C under static conditions. After incubation, the loosely attached cells were thoroughly washed three times with sterile Phosphate-buffered saline (0.01M PBS, pH 7.4). Subsequently, 50 µl of the CS, OM, or CS@OM at sub-inhibitory concentrations were mixed individually with 50 µl of Mueller-Hinton II cation-adjusted broth and added to the washed wells. The plate was incubated at 37 °C under static conditions for 24 h. Afterward, the plate was washed thrice with 200 µl sterile PBS, dried at 60 °C for 1 h, and stained with CV (Sigma-Aldrich, France) (0.1 % w/v) for 15 min. The plate was subsequently rinsed three times with dist. water to eliminate the excess stain, followed by a de-staining process using acetic acid (30 % *v/v*). Finally, the absorbance was measured at OD_{596} nm. The results are reported as mean values ± SD (n = 3).

The biofilm eradication (%) was calculated as follows:

$$\text{Biofilm eradication (\%)} = \frac{\text{OD negative control - OD treatment}}{\text{OD negative control}}$$

2.5. Detection of mechanisms of action of CS@OM

2.5.1. Time-kill curves

The most potent combinations of CS@OM demonstrating a synergistic effect (FICI ≤ 0.5) against the bacterial isolates in checkerboard assay were further subjected to a time-kill assay to evaluate their bactericidal activity (Ivanova *et al*., 2018). In summary, 200 µl of overnight-grown bacteria were diluted in sterile PBS (0.01 M, pH 7.4) to a

concentration of ~ 5.10^5 cfu/ ml. Subsequently, 200 µl of CS (sub-MIC), OM (sub-MIC), and CS@OM (sub-MIC) were added to the bacterial suspension, followed by incubation at 37 °C at 150 rpm. The surviving bacteria were counted at time 0, 1, 2, 4, 8, and 24 h using the drop plate method (Ivanova *et al*., 2018). Untreated bacteria in PBS were used as a negative control. The results are reported as mean values ± SD (n = 3).

2.5.2. Inner membrane permeability

The bacterial inner membrane permeability was assessed using O-nitrophenyl-β-D-galactoside (ONPG) (Wijesundara *et al*., 2021). Initially, bacterial suspensions were diluted in sterile PBS (0.01 M, pH 7.4) to a concentration of ~ 5×10^5 cfu/ ml. Subsequently, 20 mM/ ml ONPG (Thermo Scientific, France) were added to the bacterial suspension along with CS (sub-MIC), OM (sub+MIC), or CS@OM (sub-MIC). The samples were incubated at 37 °C at 150 rpm. The production of O-nitrophenol was monitored by absorbance measurement at OD410 nm at 0, 1, 2, and 4 h of incubation. Untreated bacteria were used as a negative control. The results are reported as mean values ± SD (n = 3).

2.5.3. Cell membrane integrity

Bacterial inocula were diluted in PBS (0.01 M, pH 7.4) to a concentration of ~ 5×10^5 cfu/ ml and treated with CS (sub-MIC), OM (sub-MIC), or a combination of CS@OM (sub-MIC). Untreated bacteria were used as a negative control. Subsequently, the samples were incubated at 37 °C for 24 h. Afterward, the samples were filtered using 0.2 µm pore size filters (Nalgene™ Sterile Syringe Filters, Thermo Scientific™, France) and centrifuged (10,000 g, 5 min.). The absorbance of the supernatants was subsequently measured at 260 nm and 280 nm to determine the nucleic acids (DNA, RNA) and proteins concentration, respectively (Wijesundara *et al*., 2021). The results are reported as mean values ± SD (n = 3).

2.6. Drug resistance development

To investigate the potential of CS@OM combination on preventing drug resistance development in bacterial isolates following 14-d of exposure to stand-alone CS or CS@OM, a resistance development assay was performed according to the method adopted by Morena *et al*., (2022). In brief, MICs of CS and CS@OM were initially determined according to the recommendations of CLSI. (2020). The next day, the wells exhibiting bacterial growth at the highest drug concentration were selected and diluted in Mueller-Hinton II cation-adjusted broth at a ratio of 1:50 (*v/v*). A fresh MIC assay was performed, and this procedure was iterated over a span of 14 d while recording any changes in the MIC values of the treatments.

2.7. Statistical analysis

All recorded data included the corresponding means and standard deviation (± SD). A one-way analysis of variance (ANOVA) was initially performed, followed by either a post-hoc Tukey's test or unpaired two-tailed Student's t-test, as appropriate. Significance levels were set at p-values less than 0.05 (*), 0.01 (**), and 0.001 (***), denoting different levels of significance in the statistical evaluation.

3. Results and Discussion

3.1. Antibacterial potential of OM and CS

The antibacterial activity of CS@OM against the eight bacterial isolates was tested using broth microdilution method. Accordingly, OM demonstrated a MIC range of 0.9 to 31.2 µg/ ml, while CS showed a MIC range of 4 to 32 µg/ ml against the eight tested isolates. Notably, all isolates displayed MBCs equivalent to their respective MICs for both CS and OM (Table 2). It is important to note that all the eight isolates were classified as colistin-resistant (CS_R) due to their MICs > 2 µg/ ml, according to CLSI. (2020) breakpoint's. The antibacterial activity of OM EO primarily originated from its major constituents, including terpinen-4-ol, α-terpinene, α-terpinol, α-pinene, and *p*-cymene, among others (Amor *et al*., 2019; Sabiha *et al*., 2023).

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CS and OM EO tested against eight clinical bacterial isolates

Isolate code	OM\MIC (µg/ ml)	OM\MBC (µg/ ml)	CS\MIC (µg/ ml)	CS\MBC (µg/ ml)
TT-7	31.2	31.2	4	4
N67	15.6	15.6	32	32
TT-82	7.8	7.8	32	32
TT-18	31.2	31.2	8	8
TT-25	7.8	7.8	16	16
TT-74	15.6	15.6	4	4
O48	7.8	7.8	32	32
O83	0.97	0.97	32	32

Numerous investigations conducted on Moroccan *O. majorana* essential oil and from various regions of the world have consistently demonstrated that these major components are responsible for *O. majorana* oil's antibacterial potential (Della *et al*., 2019; Sabiha *et al*., 2023). However, it is believed that all the constituents of the essential oil work were in synergy to express an improved antibacterial activity (Paudel *et al*., 2022).

For colistin, its main mechanism of action against GNB involves the electrostatic interactions between the positively charged amino groups of CS and negatively charged phosphate groups of the lipid A [a part of lipopolysaccharides (LPS)] of GNB. This interaction competitively displaced calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations, which are responsible for maintaining the bacterial outer membrane integrity. This displacement resulted in a cation-cation repulsion (*i.e*., colistin^{+}, Ca^{2+}/Mg^{2+} repulsion), leading to membrane integrity disruption and ultimately bacterial death (Andrade *et al*., 2020; Aftab *et al*., 2024). However, in our study, several mechanisms could explain resistance of the bacterial isolates to colistin (MIC >2 µg/ ml). Until recently, it is believed that resistance to colistin could only occur through chromosomal mutations. However, identification of the *mcr-1* gene in China in 2015 showed that resistance could also be acquired through transferable plasmids that spread between the different bacterial species (Liu *et al*., 2016). *Mcr*-like genes (*mcr*-1 to *mcr-10*) encode for a phosphoethanolamine transferase, an enzyme that catalyzes the addition of a phosphoethanolamine (pEtN) moiety to LPS of the bacterial outer membrane, reducing the affinity of colistin to its target, thereby leading to CS_R (Andrade *et al*., 2020; Hussein 2021). Interestingly, our study revealed that 2 out of 8 isolates (TT-7 (*E. coli*) and O48 (*K. pneumoniae*) carried the *mcr-1* gene (Table 1). However, for the rest of the bacterial isolates (N67, TT-82, TT-18, TT-25, TT-74, and O83), other mechanisms of resistance might be in action, including chromosomal mutations in the two-component systems (TCS) *PmrAB* and *PhoPQ*, and *arnBCADTEF* or *mgrB* genes. Similar to *mcr*-like genes, these mutations encode for modification of LPS of the bacterial membrane that becomes no longer able to bind with colistin, thus developing resistance to colistin (Andrade *et al*., 2020). Occasionally, resistance to colistin may stem from an inhibition of lipid A

biosynthesis gene expression (*lpxA*, *lpxC*, or *lpxD*), which leads to resistance to colistin attributed to the absence of lipid A (Andrea *et al*., 2024). In addition, colistin resistance can also be influenced by overexpression of the efflux pumps. Furthermore, a strong correlation has been established between biofilm formation and antibiotic resistance, providing additional insights into the resistance observed in the tested bacterial isolates against colistin (Dortet *et al*., 2016; Andrade *et al*., 2020).

3.2. Two-dimensional checkerboard assay

A checkerboard assay was employed to determine the ideal combination of colistin and *O. majorana* oil concentrations, which exhibited better synergistic activity between the two compounds, resulting in higher antibacterial potency (Wickremasinghe *et al*., 2021). Accordingly, results of CS, OM, and their combination (CS@OM) against the eight CS_R isolates using checkerboard assay are summarized in Table (3). For a better comprehensive understanding of the interpretation process of the checkerboard assay results, an illustrative example is provided in Fig. (1). In this example, the combined therapy involving CS@OM overturned colistin resistance effectively, resulting in a reduction of colistin's MIC from 4 µg/ ml to 1 µg/ ml in the *mcr-1*-positive isolate (TT-7), as evidenced by the well labeled as SYN in the 96-well plate (Fig. 1).

Similarly, for the other isolates, the checkerboard assay results showed that combination of CS@OM significantly exhibited synergistic activity (FICI index ≤ 0.5, Table 3) against 5 out of 8 tested isolates, including two *mcr-1* positive isolates (TT-7 and O48). Accordingly, the CS MICs decreased by 4-32-fold for these isolates in the presence of OM. On the other hand, an additive activity (0.5 < FICI ≤ 1) was observed in the other 3 isolates (Table 3). Interestingly, no indifferent (FICI ≤ 4) or antagonistic effects (FICI > 4) were detected between these two compounds. Explanation of the synergy between CS and OM will be discussed in detail in the following sections of this study.

3.3. Biofilm eradication

Gram-negative bacteria are known for their ability to form biofilms. A biofilm is a community of bacteria that is enclosed within a self-produced matrix made of exopolysaccharide (EPS), lipids, proteins, and DNA. Biofilm formation is often associated with different host tissues and medical devices such as catheters, ventilators, and contact lenses. Removing established biofilms is challenging as the microbial cells are trapped inside a complex structure that serves as a protective shield against the antibacterial agents (Roy *et al*., 2018; Swedan and Aldakhily, 2024). Therefore, for antibacterial agents to be effective, they must penetrate the EPS matrix and reach the surface-attached cells. For this purpose, the ability of the combination CS@OM to eradicate the pre-existing biofilms using CV assay was tested (Ivanova *et al*., 2020).

At sub-MIC concentrations, CS individually eradicated the biofilm biomass of the isolates recording values ranging from approximately 14 % (against TT-25) to about 44 % (against TT-7). Simultaneously, stand-alone OM exhibited notable biofilm eradication potential, showing efficacy rates ranging from around 46 % (against TT-25) to approximately 61 % (against O83) (Fig. 2). Nevertheless, the combined CS and OM (CS@OM) revealed high capability for biofilm eradication compared to using stand-alone CS or OM. Consequently, the biofilm eradication rates significantly increased; ranging from approximately 52 % (against O83) to about 82 % (against O48).

In this study, the antibiofilm activity of CS@OM combination could be mainly related to OM EO. Indeed, previous studies highlighted that OM EO is successfully able to inhibit biofilm formation in both Gram-negative and Gram-positive bacteria (Semiz *et al*., 2018; Ben Abdallah *et al*., 2020). The hydrophobic nature of the essential oil components helps in destabilizing and infiltrating through the biofilm matrix.

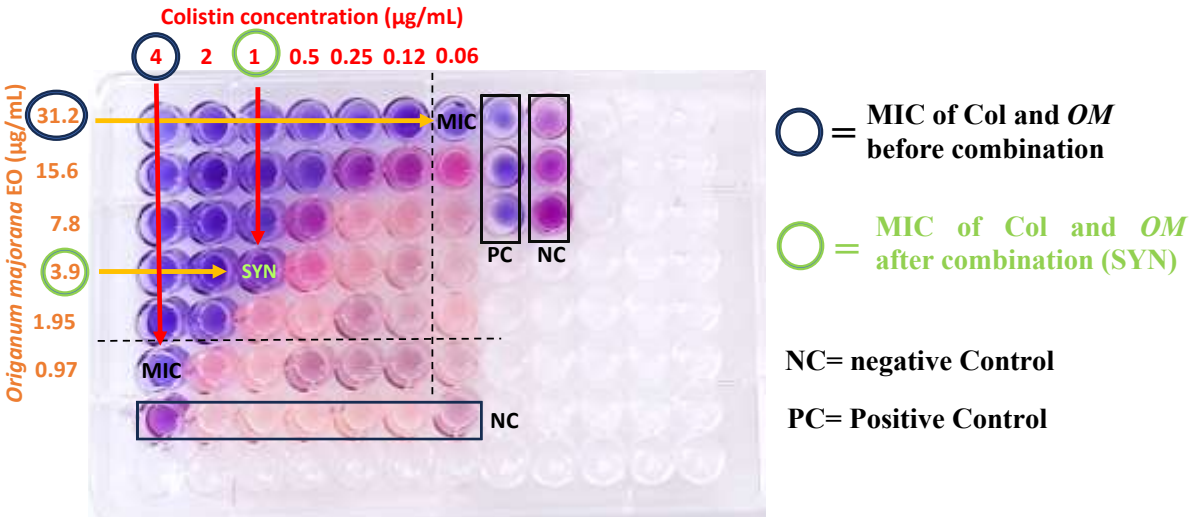

Fig. 1: Example of checkerboard assay performed using CS and OM EO in combination against the CS_R *E. c*oli (TT-7) harboring *mcr-1* gene

Table 3: Minimum inhibitory concentrations (MICs) of stand-alone CS and OM EO and their combination (CS@OM) against the selected bacterial isolates

Isolates code	Monotherapy MIC		Combination MIC		Reduction MIC (X-fold)		FICI Index	Interpretation
	CS (µg/ ml)	OM (µg/ ml)	CS (µg/ ml)	OM (µg/ ml)	CS	OM		
TT-74	4	15.6	2	7.8	2	2	1	Additive activity
TT-82	32	7.8	4	3.9	8	2	0.75	Additive activity
N67	32	15.6	16	15.6	2	2	1	Additive activity
TT-18	8	31.2	2	3.9	4	8	0.5	Synergistic activity
TT-25	16	7.8	1	1.97	16	8	0.31	Synergistic activity
TT-7	4	31.2	1	3.9	4	8	0.37	Synergistic activity
O48	32	7.8	1	0.48	32	16	0.13	Synergistic activity
O83	32	0.97	2	0.24	16	4	0.31	Synergistic activity

Where; FICI: Fractional inhibitory concentration index

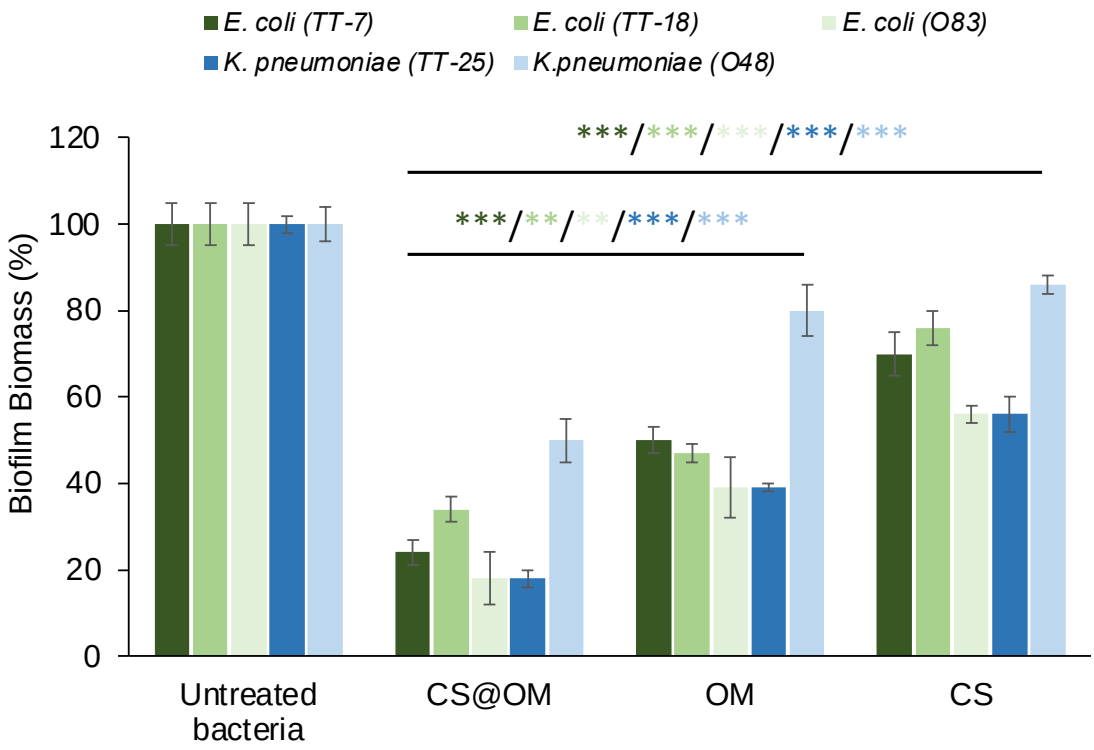

Fig. 2: Biofilm eradication using crystal violet assay, involving biofilm biomass (%) of the bacterial isolates following treatment with CS, OM, and CS@OM

Once inside the biofilm matrix, the EOs exert their intrinsic antibacterial activity, causing sessile cells to detach from the surfaces and kill the planktonic bacterial cells (Dos Santos *et al*., 2017). We speculate that destabilization of the biofilm matrix by OM will allow CS to infiltrate through, thereby exerting its bactericidal effect at lower dosages against the planktonic cells of the biofilm (Klinger-Strobel *et al*., 2017). This explains the CS enhanced activity against biofilms when used in combination with OM EO. Interestingly, terpinen-4-ol; a major component of *Majoaran* oil, downregulates the expression of genes responsible for biofilm adherence to the surfaces, reduces EPS synthesis in bacteria, and interrupts cell-to-cell communication among bacteria (quorum sensing signals), thereby impeding their ability to produce biofilms (Bose *et al*., 2020; Zhao *et al*., 2021). The demonstrated efficacy of the CS@OM combination in efficiently eradicating the preexisting bacterial biofilm serves as an additional justification for the improved antibacterial potential of colistin, specifically since biofilm formation is often associated with antibiotics resistance such as resistance to colistin (Cepas *et al*., 2019).

3.4. Mechanisms of action of CS@OM

3.4.1. Time-kill curves

A time-kill assay was carried out to assess viability of the treated bacteria and the time required for treatments to manifest their bactericidal activity. For this purpose, five isolates were selected based on the demonstrated synergistic activity observed between colistin and *O. majorana* oil (CS@OM) in the checkerboard assay (Table 3). The results showed that combination of CS@OM rapidly reduced bacterial load by ~4 $\log_{10}$ cfu/ ml within 1-2 h of incubation, with complete eradication achieved after 4-8 h (Fig. 3).

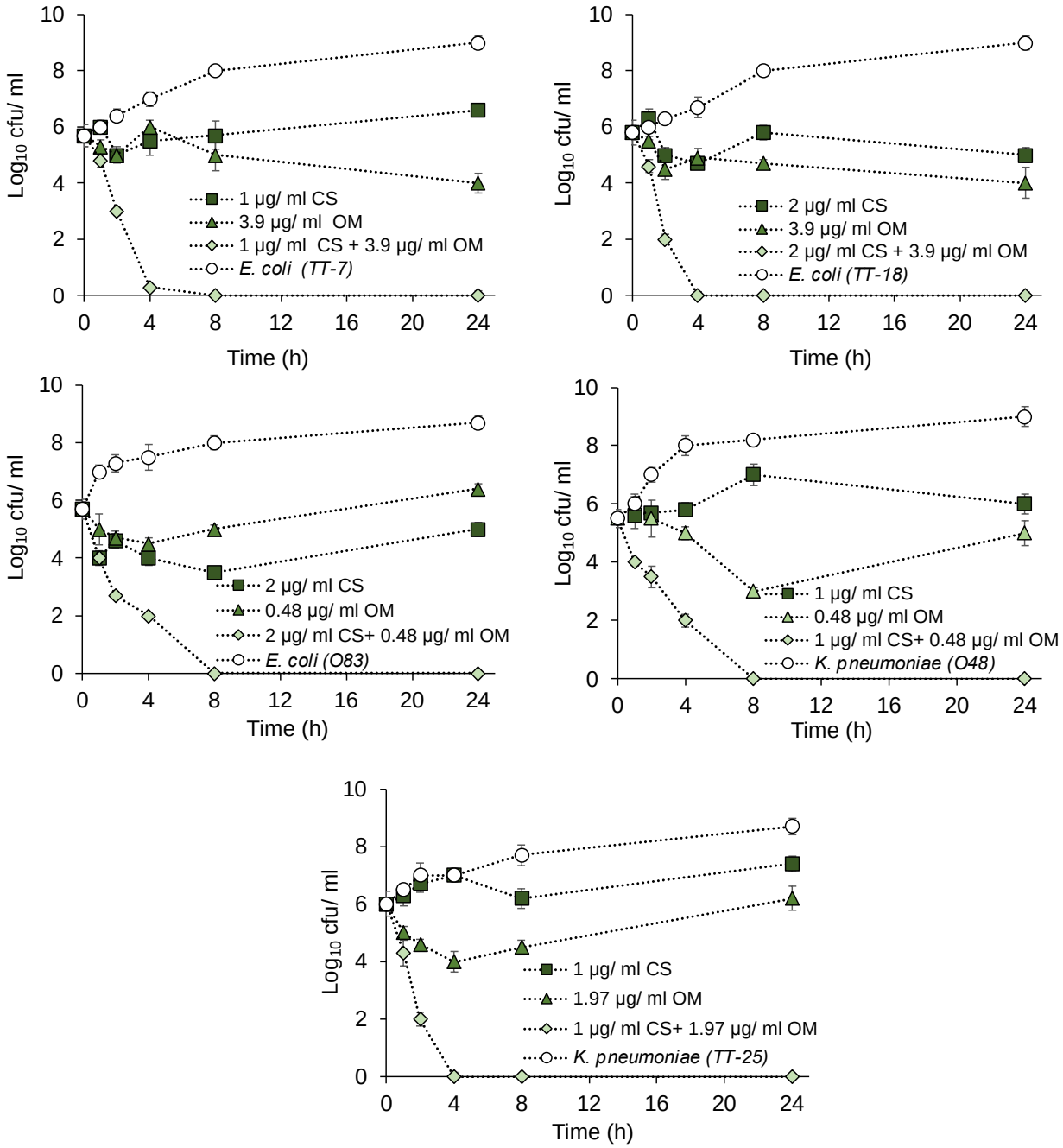

Fig. 3: Time-killing curves of CS@OM (colistin+ *O. majorana*) on the selected bacterial isolates. The isolates were incubated with sub-MIC concentrations of CS@OM (colistin+ *O. majorana*), CS (colistin), and OM (*O. majorana*). The developing bacteria were counted after 1, 2, 4, 8 and 24 h of incubation

This combination also maintained its bactericidal effect even after 24 h, resulting in a significant reduction of ~9 $\log_{10}$ cfu/ ml compared to the untreated bacteria. In contrast, use of *O. majorana* oil or colistin independently demonstrated minimal to negligible inhibition of bacterial growth, which persisted even after 24 h of incubation.

3.4.2. Inner membrane permeability

The cytoplasmic membrane serves as a crucial barrier, selectively regulating the movement of specific molecules into and out of the bacterial cells. Moreover, it plays a pivotal role in preserving the cell's structure and enabling cell-to-cell communication (Murínová and Dercová, 2014; Ada *et al*., 2020). For this purpose, the inner cell membrane permeability of the treated bacteria was assessed using O-nitrophenyl-β-D-galactoside as a substrate for the cytoplasmic β-galactosidase enzyme, which would leak through the inner membrane upon its disruption. The primary function of this enzyme is to break down lactose into glucose and galactose (Gravel *et al*. 2017; Jinxin *et al*., 2024). The results demonstrated that when bacteria were exposed to a combination of CS@OM, the absorbance values (OD_{410} nm) were significantly higher than when exposed to either stand-alone form (CS or OM) (Fig. 4). Moreover, the absorbance gradually increased over time, peaking at 4 h of exposure to the treatment, indicating a time-dependent effect of the treatments. These findings suggest that combination of CS and OM caused damage to the bacterial inner membrane, resulting in leakage of the cytoplasmic components, including the β-galactosidase enzyme.

Leakage of β-galactosidase from bacterial cells into the extracellular compartment can be attributed to the combined effect of CS and OM EO. These compounds target distinct paths within the bacterial cells, complementing each other in their modes of action (Gomes *et al*. 2020; Sabnis *et al*., 2021). However, according to the MIC results, it was observed that the bacterial isolates employed in this study displayed resistance to colistin, implying that the binding affinity of colistin to LPS was reduced due to the aforementioned enzymatic modification of LPS (Andrade *et al*., 2020; Hussein, 2021). Therefore, the following synergistic action scenario can be outlined: OM EO's hydrophobic components allow it to interact easily with the lipid fraction of the bacterial outer cell membrane, altering the membrane's fluidity and destabilizing its structure, which enables OM EO to reach the cytoplasmic membrane (CM) (Haghighatpanah *et al*., 2022). Disturbance of the membrane structure by OM may facilitate CS's binding to LPS of both the outer and inner bacterial cell membranes by inserting its hydrophobic terminal acyl fat chain, thereby causing further expansion of the membrane and leakage of the intracellular components such as β-galactosidase (Hazime *et al*., 2022). Destabilization of the bacterial inner membrane is directly linked to a decrease in adenosine triphosphate (ATP) levels within the cell, since ATP synthesis in bacteria takes place through the electron transport chain embedded within the cytoplasmic membrane (Bouyahya *et al*., 2018). In addition, disruption of the inner membrane can deplete the small molecules such as K^+, Na^+, and H^+ ions, which are critical for proper membrane function, enzyme activity maintenance, and normal metabolic processes (Stautz *et al*., 2021). Hence, even slight variations in ion homeostasis can adversely affect cell metabolism and bacterial survival.

3.4.3. Cell membrane integrity

To further assess the extent of cell wall damage caused by CS@OM, leakage of cytoplasmic contents was quantified using absorbance at 260-280 nm to detect DNA/RNA and proteins in the supernatant of the treated bacteria (Wijesundara *et al*., 2021). Accordingly, a significant difference in absorbance at 260-280 nm was detected when bacteria were treated with CS@OM, compared to stand-alone CS or stand-alone OM (Fig. 5).

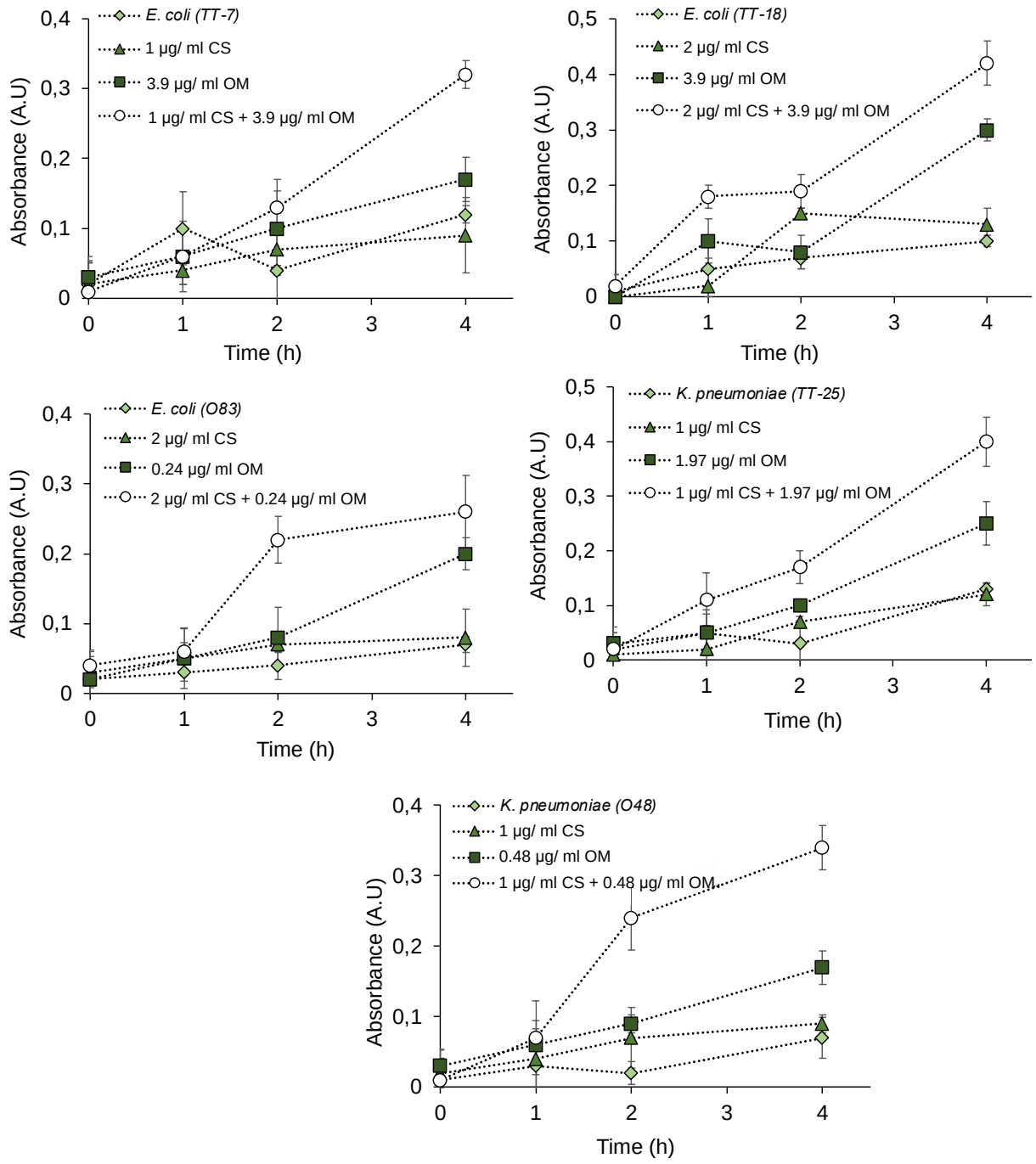

Fig. 4: Cell wall inner membrane permeability after isolates treatment with sub-MIC concentrations of CS@OM, CS, and OM, determined by cytoplasmic β-galactosidase activity using O-nitrophenyl-β-Dgalactoside (ONPG) as a substrate, and measured with an increase in absorbance at 410 nm.

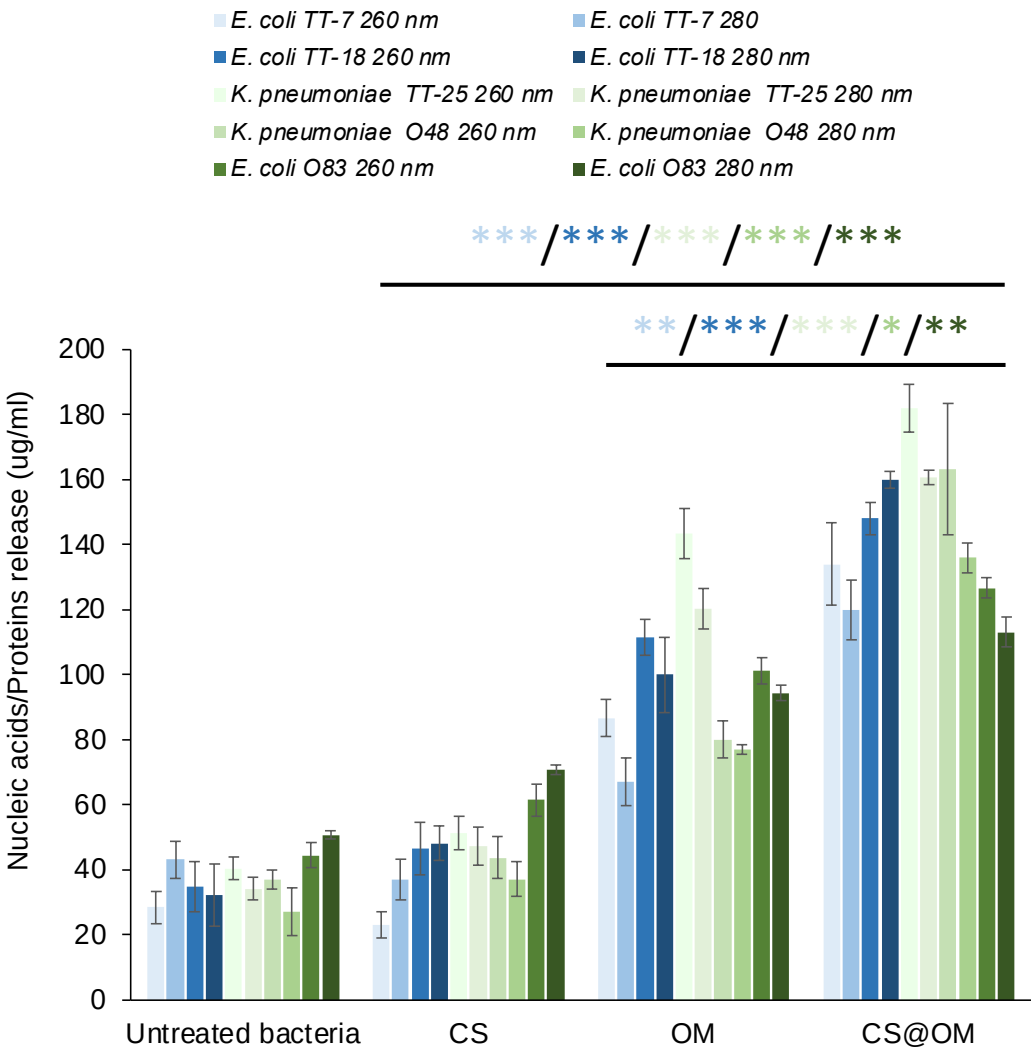

Fig. 5: Cell membrane integrity assay. Disruption of cell membrane integrity detected by the cytoplasmic release of DNA/RNA and protein, assessed by absorbance measurement at 260-280 nm after bacterial treatment with CS@OM, CS, and OM for 24 h

These results indicated that more pronounced damage occurred to the cell membrane by CS@OM, which led to the leakage of large molecules such as proteins and DNA/ RNA into the supernatant. These findings provide further confirmation of the ability of CS@OM to degrade bacterial cell walls and release cytoplasmic contents. In addition to the previously discussed mechanisms reported by Haghighatpanah *et al*., (2022); Hazime *et al*., (2022), it is believed that colistin can trigger the production of reactive oxygen species (ROS) in bacteria. These generated ROS can induce oxidative damage to the cell membrane lipids, protein degradation, and fragmentation of genomic DNA (Yu *et al*., 2017; Maslovska *et al*., 2023). Consequently, this initiated additional harm to the bacterial cell membrane; primarily composed of lipids, resulting in significant leakage of the inner cellular components.

3.5. Drug resistance development

Acquiring resistance to antibiotics is easy for the bacteria since most conventional antibiotics target a specific pathway within the microorganism. To overcome this issue, researchers are working on designing new antibacterials that exhibit multitude of mechanisms of action. Acknowledging that a standard antibiotic treatment lasts from 7 to 14 d (Kristoffersen *et al*., 2009), our bacterial isolates were exposed to sub-lethal doses of CS and CS@OM for a 14-d period, diligently monitoring and documenting any changes in their MICs. After 14 d of exposure to stand-alone CS, the isolates exhibited increased resistance ranging from 8 to 128 times, which was translated by an increase in their MICs by 8-128-fold (Fig. 6). Similar results were observed in a previous study conducted by Safronova *et al*., (2022) that tested the ability of MDR *E. coli* to develop resistance against polymyxin B after 24-d exposure. However, no change in MIC values was observed when the isolates were treated with a combination of CS@OM during the same period; with the exception of isolate TT-43, where the MIC expressed a two-fold increase. The CS@OM's capacity to prevent resistance development might be attributed to the variety of mechanisms of action that both CS and OM exhibit, including antibiofilm activity, bacterial membrane damage, down-regulating gene expression, ROS production, ATP synthesis inhibition, and efflux pump inhibition.

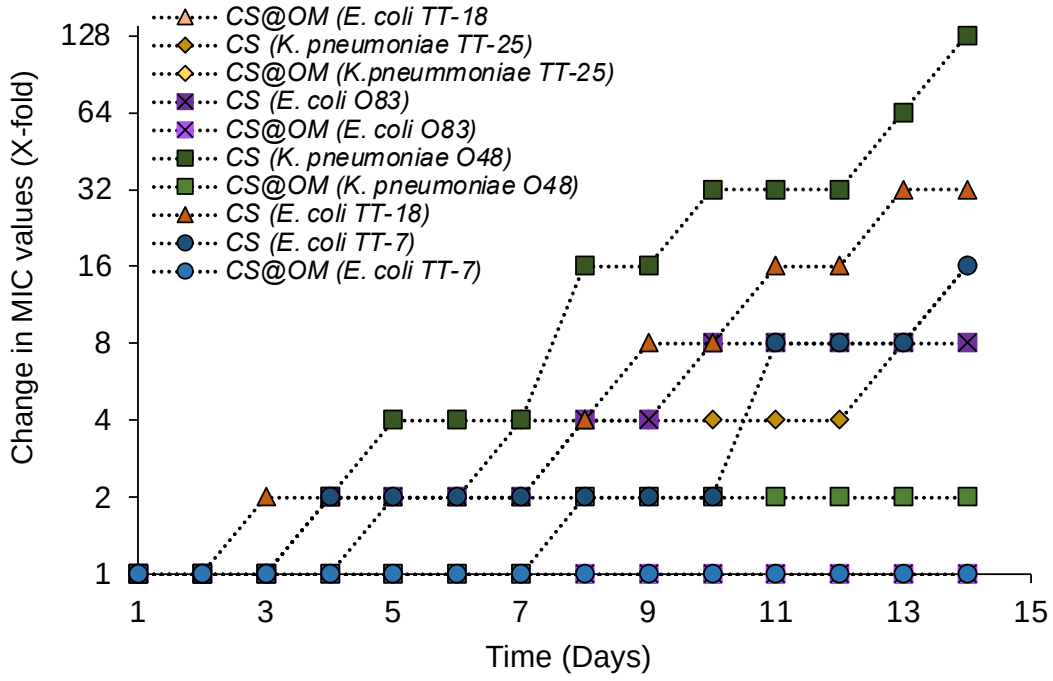

Fig. 6: MIC value change for bacterial isolates after 14-d exposure to stand-alone CS and CS@OM

Conclusion

Gram-negative bacteria, such as *E. coli* and *K. pneumoniae*, have demonstrated a remarkable ability to develop resistance against antibiotics. They are commonly associated with both community and hospital-acquired infections. Due to the lack of new antibiotics as well as inadequate activity of the existing antibiotics, the emergence of multi-drug resistant GNB has called for an urgent need to develop strategies to reinforce efficacy of the current antibiotics. With this aim, we conducted a checkerboard test to determine the probable synergistic activity of the combination between colistin and *O. majorana* EO. Additionally, this combination will further aid in improving the effectiveness of colistin against colistin-resistant isolates. Interestingly, the combination CS@OM demonstrated improved antibacterial and antibiofilm activities against the eight isolates used in this study. The heightened activity of this combination was attributed to the synergistic action of both colistin and *O. majorana* EO. Meanwhile, *O. majorana* essential oil effectively degraded the biofilm matrix of the isolates, which facilitated the contact of colistin with the planktonic bacterial cells within the biofilm matrix. Moreover, the combination CS@OM displayed bactericidal activity through bacterial membrane disturbance, release of the internal cell contents, and cell death. Finally, CS@OM efficiently avoided drug resistance development in the isolates after 14-d exposure. Given the combination's heightened antibacterial activity and capacity to eradicate biofilms, CS@OM could be further used as a treatment for biofilm-related infections and colistin-resistant GNB infections.

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Conflict of interests

The authors have no relevant financial conflicts of interest to disclose.

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Ethical approval

Non-applicable.

Author's Contributions

Zine El Abidine Bzazou EL Ouazzani: Conceptualization and Methodology, Formal analysis, Investigation, Resources, Writing-Original Draft. Houda Benaicha: Conceptualization and Methodology, Writing-Review & Editing. Laila Reklaoui: Formal analysis, Investigation. Said Barrijal: Conceptualization and Methodology, Resources, Writing-Review & Editing, Supervision.

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