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Evaluation of Azithromycin and Fenugreek Oil as Anti-virulence Agents against *Stenotrophomonas maltophilia* MultiDrug Resistant Clinical Isolates

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STENOTROPHOMONAS maltophilia is a gram-negative opportunistic pathogenic bacterium that is associated with hospital- and community-acquired infections. It has a set of virulence factors, such as biofilm formation and extracellular enzymes, that are mostly regulated via quorum sensing (QS) systems. Azithromycin (AZM) is a macrolide that is well known for its anti-virulence effects, including anti-QS and antibiofilm effects. Additionally, some spice essential oils have been reported to inhibit bacterial virulence. This study evaluated the effect of AZM and Fenugreek Oil (FO), a spice essential oil from Fenugreek seeds, against some virulence factors of multidrug-resistant *Stenotrophomonas maltophilia* clinical isolates. Both AZM and FO showed significant inhibitory effects against protease activity, where all tested isolates showed 100% loss of the halo zone formed in skimmed milk agar test with AZM and a 25 to 35% reduction in the zone with FO. A mean reduction in the interstitial surface area of 34.4% and 35.5% was detected with AZM and FO, respectively, in the twitching motility assay. While AZM showed a significant effect in reducing biofilm formation by *S. maltophilia* isolates (mean inhibition of 49.7%), the reducing effect of FO (18.5%) was not significant. Genotypically, exposure of *S. maltophilia* clinical isolates to AZM and FO significantly reduced the expression of protease-encoding genes (*stmPr1, stmPr2 and StmPr3*) and a quorum sensing gene (*rpfC*).

Keywords: Anti-virulence agents, Azithromycin, Biofilm, Fenugreek oil, Quorum sensing, *Stenotrophomonas maltophilia.*

Introduction

Stenotrophomonas maltophilia (*S. maltophilia*) is a gram-negative multidrug-resistant (MDR) bacterium with a high global clinical concern. It is most associated with respiratory, bloodstream and several serious infections in humans, especially in immunocompromised individuals. It is known as an important nosocomial pathogen and as a community-acquired pathogen. The World Health Organization classified *S. maltophilia* as one of the leading MDR organisms in hospital settings. *S. maltophilia* has inherent and acquired resistance to a variety of antimicrobial agents; therefore, its treatment is considered a great challenge (Guyot et al., 2013; Brooke, 2021).

S. maltophilia has many virulence factors related to its pathogenicity. These factors may be extracellular (mainly enzymes, including hemolysins, proteases and siderophores) and cell-associated virulence factors (lipopolysaccharide, fimbriae and flagella) (Trifonova & Strateva, 2019). In addition, biofilm formation provides a great advantage for bacterial virulence. It can be formed on both host tissues and abiotic surfaces and can promote high resistance to antibacterial agents and escape the host immune system (Brooke, 2021).

S. maltophilia uses the quorum sensing (QS) system to coordinate the expression of a large set of genes through interactions between QS signaling

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molecules and receptors after reaching a certain cell count. For *S. maltophilia,* QS is regulated via 2 pathways: through the signaling molecule N-acyl homoserine lactones and diffuse signal factor QS (DSF-QS) (Adegoke et al., 2017; Mojica et al., 2022). DSF synthesis relies on the *rpf*F gene within the rpf operon for virulence factors regulation, including bacterial motility, biofilm formation, and extracellular proteases (Adegoke et al., 2017).

Recently, anti-virulence therapy has provided a promising policy to combat different bacterial infections. These drugs affect the bacterial-host interactions in different ways and hence disarm the bacteria and so minimize host damage (Dehbanipour & Ghalavand, 2022). Consequently, the potential of the organism to cause the disease is decreased and gives the upper hand for the immune system to resolve the infection. Since these agents are not bactericidal, selection pressure is not generated, which may decrease the emergence of resistance (Calvert et al., 2018). One of the strategies of anti-virulence therapy is interrupting the quorum sensing system, which in turn inhibits virulence-related gene expression and biofilm formation (Chbib, 2020).

The macrolide azithromycin is one of the approved antibiotics that is recently known for its anti-virulence effects and anti-quorum sensing effects at subinhibitory concentrations against *Staphylococcus aureus* (Ikemoto et al., 2020) and *Pseudomonas aeruginosa* (Elsheredy et al., 2021). On the other hand, some spice essential oils have been reported to inhibit bacterial virulence (QS, motility, toxin production and biofilm formation). These effects were mainly related to the different phytochemical compounds of the oils. They can be considered ideal anti-virulence agents because they are mainly extracted from natural, available and safe spices (Kim et al., 2016; Zhang et al., 2020). Fenugreek oil (FO), which is derived from fenugreek seeds and has antioxidant compounds and unsaturated fatty acids, is known for its antibacterial properties (Hadi & Mariod, 2022). Nevertheless, its effect on bacterial virulence factors has not yet been investigated.

This study aimed to evaluate the effect of AZM and FO against some virulence factors of MDR *S. maltophilia* clinical isolates using phenotypic and genotypic techniques. To the best of our knowledge, this is the first study to evaluate the anti-virulence effect of AZM against MDR clinical isolates of *S. maltophilia* and to assess FO for its anti-virulence effects.

Materials and Methods

Bacterial isolates

A total of 50 *S. maltophilia* isolates were collected from different clinical samples, including blood samples, sputum samples, and BAL (bronchoalveolar lavage) samples. The clinical samples were obtained from the microbiological laboratories of three different hospitals in Alexandria, Egypt. Conventional biochemical methods were applied for initial identification of all the isolates (Tille, 2015), which were then confirmed by Vitek-2 (bioMerieux, France). *S. maltophilia* ATCC 13637 (Oxoid, London, UK) was used as a reference strain tested under the same conditions as the clinical isolates.

Antibacterial susceptibility test

The disc diffusion method was used to assess the antibacterial susceptibility of all isolates. The discs used (Oxoid, London, UK) were minocycline (MIN, 30µg), levofloxacin (LEV, 5µg), ceftazidime (CAZ, 30µg), tetracycline (TE, 30µg) and trimethoprim-sulfamethoxazole (SXT, 1.25\23.75 µg) discs. Interpretation of the obtained values into sensitive, intermediate, and resistant categories was performed according to the Clinical and Laboratory Standards Institute (CLSI) recommendations 2022 (Clinical and Laboratory Standards Institute CLSI, 2022) Isolates that showed resistance to 3 or more of the tested antimicrobials were considered MDR and were used for the following investigations.

Determination of minimum inhibitory concentration (MIC) for each of AZM and FO against the MDR clinical isolates using broth microdilution method

The MICs of AZM (Amriya Pharmaceuticals, Egypt) and FO (Purity Company, Egypt) against the *S. maltophilia* clinical isolates and *S. maltophilia* strain ATCC 13637 were determined using the broth microdilution method (Tille, 2015). AZM and FO were solubilized using dimethyl sulfoxide (DMSO), producing a stoke solution of 100 mg/ml and 20µL/mL, respectively. Each isolate was then examined in a microtiter plate against each AZM and FO (in nutrient broth with twofold serial dilutions) at concentrations starting from 250µg/mL and 10µL/mL, respectively. The inoculum of each isolate was adjusted to 1.5×10^8 CFU/ml (OD_{600} 0.12–0.13) and then diluted to obtain

5×10^5 CFU/mL as the final concentration. The test was performed in duplicate for confirmation. Sub-MIC was considered as the concentration just below the MIC (½ MIC) and was used in all further investigations.

Phenotypic detection of virulence factors

Biofilm formation assay

S. maltophilia isolates were tested for their biofilm production ability using the microtiter plate method with slight modifications (Hassan et al., 2011). Briefly, each isolate was incubated in sterile trypticase soy broth (TSB) in a glass tube (with and without AZM or FO) overnight at 37°C with shaking. Then, the culture turbidity was adjusted to 1×10^6 CFU/ml, and 200µL was transferred to 3 wells of a 96-well sterile flat-bottomed microtiter plate. After incubation of the plates (24h at 37°C), the well contents were decanted and washed using phosphate-buffer saline (PBS) (pH 7.2). The formed biofilm was fixed using 2% sodium acetate solution and then washed with PBS. Biofilms were stained with 1% crystal violet solution for 20min and washed with PBS. Then, the stain was dissolved using 95% ethanol. The optical density (OD) at 620nm was read on a microtiter plate reader. The assay was performed in triplicate to calculate the average OD. A negative control (TSB without bacterial suspension) was included, and its average absorbance was measured and denoted as the cutoff optical density (ODc). The isolates were classified according to their OD to: nonbiofilm producer (isolate OD ≤ ODc), weak biofilm producer (ODc < isolate OD ≤ 2 ODc), moderate biofilm producer (2ODC < isolate OD ≤ 4 ODC) and strong biofilm producer (isolate OD > 4 ODC). Additionally, the percentage inhibition of biofilm formation by AZM or FO was calculated using the following formula: $(1 - OD_{620}/ODc_{620}) \times 100$ (Jadhav et al., 2013).

Twitching motility assay

The twitching motility assay was performed according to Rashid and Kornberg (2000) with some changes (Rashid & Kornberg, 2000). The test was performed for each isolate in the absence and presence of AZM or FO. Each isolate in each condition was tested in duplicate. Briefly, twitching plates of soft agar were inoculated with bacteria from overnight TSB cultures using a sterile toothpick to the Petri dish bottom and then incubated at 37°C for 24h. The plates were examined for the interstitial zone, i.e., the haze zone of growth at the interface between the soft agar and the Petri plate, as seen by examining the plate against a light source. The interstitial zone surface area was calculated according to Turnbull and Whitchurch (Turnbull & Whitchurch, 2014). Then, the percentage change in the interstitial zone surface area by AZM or FO was estimated.

Protease activity assay

The protease activity was investigated quantitatively on 1% skimmed milk agar plates where 40µL of each isolate was inoculated in wells cut by sterile cork-borer (of 7mm diameter). The plates were incubated for 24h at 37°C, and the developed halo-zone diameter was measured (Alcaraz et al., 2021). The test was performed in duplicate for each isolate without and with AZM or FO. The percentage change in the halo-zone diameter in the presence of AZM or FO was calculated.

Quantitative reverse transcription PCR (qRT–PCR) investigating the expression of protease genes and a QS gene

The standard strain *S. maltophilia* ATCC 13637 in addition to the five MDR isolates that revealed the most remarkable decrease in phenotypic assays by AZM and/or FO were selected. A single colony from a fresh overnight culture was inoculated into fresh LB broth in the absence or presence of AZM or FO at an initial OD_{450} of 0.15 and then incubated for 5 hours at 37°C with shaking. Cells were harvested by centrifugation, and the PureLink™ RNA Mini Kit from Invitrogen (Thermo Fisher Scientific, California, USA) was used for RNA extraction according to the manufacturer's instructions. Then Nano-drop spectrophotometer (Thomas Scientific, USA) was used to quantify and determine the quality of the total extracted RNA.

A High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems™ (Thermo Fisher Scientific, California, USA) was used for the synthesis of cDNA from 0.5µg of RNA using random hexamer primers.

qPCR amplification was achieved using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, California, USA) and the PCR thermal cycler Applied Biosystem StepOne™ instrument (Thermo Fisher Scientific, California, USA). All primers used in this study were supplied by Invitrogen (Thermo Fisher Scientific, California, USA). The primer sequences are listed in Table 1.

TABLE 1. Primers used in this study

Primer		Sequence (5'–3')	Annealing temperature in °C	Reference
*stmPr*1	Forward	GCCGAAGTCATCAACCTCTC		(Molloy et al., 2019)
	Reverse	ACACGTTGGTGTTGCTGTTG		
stmPr2	Forward	CGTGCCAGCTTCTCCAACTA	50	(DuMont et al., 2015)
	Reverse	AGGACTGTTGATGGTGCAGG		
stmPr3	Forward	ATCGACAGCACCTGCAACTAC		(Molloy et al., 2019)
	Reverse	TTCACATCGCGATAGGACAG		
*rpf*C	Forward	TGTTCGGCTGGCTGCTGT	60	(Huedo Moreno, 2015)
	Reverse	GGCCAGCACTTCGGTCAT		
rpoB	Forward	5'- AGGAAATGCTGACGGTGAAG -3'	50	(Esposito et al., 2020)
	Reverse	5'- ACGAGCACGTTGAAGGATTC -3'		

qPCR was used to determine the fold change in the expression of *S. maltophilia* protease genes (*stmPr1*, *stmPr2*, *stmPr3*) and a quorum sensing gene (*rpfC*) in the presence of AZM or FO relative to control expression in the absence of drug treatment after normalizing the data to the housekeeping gene *rpoB* (Esposito et al., 2020) using the $2^{-\Delta\Delta Ct}$ formula (Livak & Schmittgen, 2001).

Statistical analyses

Data were analyzed using IBM SPSS software version 20.0. (Armonk, NY: IBM Corp). Quantitative data are described as the range (minimum and maximum), mean, and standard deviation. To compare two groups, the chi-square test was used. If the expected count of more than 20% of the cells was less than 5, Fisher's exact and Monte Carlo correction tests were applied. The Shapiro–Wilk test was used for continuous data to test for normality. The Kruskal–Wallis test was used for abnormally distributed quantitative variables to compare more than two studied groups, while the F test (ANOVA) was used for normally distributed quantitative variables and post hoc (Dunn's multiple comparisons test) was used for pairwise comparisons. The significance of the obtained results was judged at the 5% level.

Results

The results of the susceptibility test revealed that the highest resistance was reported against ceftazidime and tetracycline (50%). Isolates were resistant to trimethoprim-sulfamethoxazole (46%), minocycline (44%) and levofloxacin (46%). On the other hand, 21 out of the 50 isolates (42%) were MDR. These MDR isolates were used for all the upcoming investigations included in the study.

MIC of AZM and FO against S. maltophilia MDR clinical isolates:

The MICs of AZM against the 21 tested MDR isolates ranged from 62 to 16µg/mL, while the MICs of FO ranged from 2.5 to 0.11 µl/ml. Concerning the *S. maltophilia* ATCC 13637 strain, the MIC of AZM was 4µg/mL, whereas the MIC of FO was 2.5µL/mL. For each isolate, the sub-MIC was considered the concentration just below MIC, i.e., ½ MIC for each of AZM and FO, which was used in the subsequent assays.

The phenotypic effect of AZM and FO on S. maltophilia virulence factors:

The effect of AZM and FO on S. maltophila biofilm formation

Out of the 21 MDR *S. maltophilia* isolates investigated for biofilm formation, 4 isolates were strong biofilm producers, and 17 isolates were moderate biofilm producers. The presence of AZM showed a statistically significant difference ($P<0.001$), where 18 isolates became weak producers and only 3 remained moderate producers. (Table 2) AZM also showed a well noticed biofilm percentage inhibition with a mean value of 49.7%. Meanwhile, FO did not show a significant effect on biofilm production but still showed a percentage inhibition with a mean value of 18.5%. The *S. maltophilia* ATCC 13637 strain was a moderate biofilm producer that became a weak producer in the presence of AZM with a percentage inhibition of 73.3% in biofilm formation, while in the presence of FO,

it remained a moderate producer but still showed a percentage inhibition in biofilm formation (39.8%).

Twitching motility assay

All the MDR *S. maltophilia* isolates showed twitching motilities on soft agar plates, where the surface area of the interstitial zone ranged from 3.8 to 5.3cm^2. In the presence of AZM and FO, there was a statistically significant difference in the interstitial zone surface area. (Table 3) In addition, each AZM and FO corresponded to a percentage decrease in the surface area of the interstitial zones, with mean values of 34.4% and 35.5%, respectively. AZM showed complete inhibition of twitching motility for the *S. maltophilia* ATCC 13637 strain, while FO caused a 19% decrease in the surface area of the interstitial zone.

Protease activity assay

Protease activity was detected for all the MDR *S. maltophilia* isolates included in the study where the diameter of the halo zone ranged from 1.2 – 1.5cm. There was complete inhibition of protease activity (i.e., 100% inhibition) with AZM. However, with FO, there was a decrease in the halo zone, where the percentage decrease ranged from 25.0 to 53.3%. (Table 4). On the other hand, the *S. maltophilia* ATCC 13637 strain did not produce protease (Fig 1).

Investigation of some virulence-related genes expression

AZM showed significant downregulation of the virulence genes *stmPr1* (0.02 ± 0.009), *stmPr2* (0.04 ± 0.008), *stmPr3* (0.014 ± 0.0005), and *rpfC* (0.025 ± 0.006). Similarly, FO showed significantly lower relative expression levels than the untreated conditions (Fig 2).

On the other hand, for the standard strain *S. maltophilia* ATCC 13637, protease genes showed a higher relative expression in the presence of AZM (by 1.5-, 2.2-, and 2.3-fold for *stmPr1*, *stmPr2 and stmPr3,* respectively) and in the presence of FO (by 2.3-, 2- and 1.4-fold for *stmPr1*, *stmPr2 and stmPr3,* respectively). For the *rpfC* QS-related gene, there was a downregulation of 14% with AZM and 10% with FO.

TABLE 2. Effect of Azithromycin and Fenugreek oil on *S. maltophilia* biofilm forming capacity

	Control (n = 21)	**AZM (n = 21)**	**FO (n = 21)**	**Test of Sig.**	**P**
Weak producer	0 (0%)	18 (85.7%)	0 (0%)	χ^2= 50.548*	^{MC}P <0.001*
Moderate producer	17 (81%)	3 (14.3%)	17 (81%)		
Strong producer	4 (19%)	0 (0%)	4 (19%)		
Sig. diff.	$^{MC}P_1$<0.001*, $^{FE}P_2$=1.000, $^{MC}P_3$<0.001*				

χ^2: **Chi square test** MC: **Monte Carlo** FE**: Fisher Exact**
P: P value for comparing between the three studied groups
P_1: P value for comparing between Alone and AZT
P_2: P value for comparing between Alone and FO
P_3: P value for comparing between AZT and FO
*: Statistically significant at P≤ 0.05

TABLE 3. Effect of AZM and FO on *S. maltophilia* twitching motility

	Alone (n = 21)	**AZM (n = 21)**	**FO (n = 21)**	**H**	**P**
Surface area of the interstitial colony (cm^2)					
Mean ± SD.	4.54 ± 0.53	2.94 ± 0.29	2.88 ± 0.30	45.709*	<0.001*
Median (Min. – Max.)	4.52 (3.80 – 5.31)	3.14 (2.54 – 3.14)	3.14 (2.54 – 3.14)		
Sig. diff.	P_1<0.001*, P_2<0.001*, P_3=0.710				

SD: **Standard deviation** H: H for **Kruskal Wallis test,** pairwise comparison between each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**
P: P value for comparing between the three studied groups
P_1: P value for comparing between **Alone** and **AZT**
P_2: P value for comparing between **Alone** and **FO**
P_3: P value for comparing between **AZT** and **FO**
*: Statistically significant at P≤ 0.05

Discussion

S. maltophilia is a gram-negative opportunistic bacterium associated with nosocomial and community-acquired infections. It is considered one of the most important MDR pathogens. Additionally, biofilm formation represents an increased challenge for *S. mailtophilia* infections (Brooke, 2021; Gibb & Wong, 2021). Anti-virulence therapy can reduce the incidence of resistant bacterial strains and limit the use of broad-spectrum antibiotics (Dehbanipour & Ghalavand, 2022). AZM is a macrolide that is well known for its anti-virulence effects, including anti-QS and antibiofilm effects (Ikemoto et al., 2020; Elsheredy et al., 2021). It is not an approved antibacterial agent for *S. maltophilia* infections and has no declared breakpoints. Additionally, some spice essential oils have been reported to inhibit bacterial virulence (Zhang et al., 2020).

TABLE 4. Effect of AZM and FO on *S. maltophilia* protease activity

	Control (n = 21)	AZM (n = 21)	FO (n = 21)	H	P
Halo zone diameter (cm)					
Mean ± SD.	1.31 0.1 ±	0 0 ±	0.78 0.08 ±	57.750*	<0.001*
Median (Min. – Max.)	1.3 (1.2 1.5 –)	0 (0 0 –)	0.8 (0.7 0.9 –)		
Sig. diff.	P_1<0.001*, P_2<0.001*, P_3<0.001*				

SD: **Standard deviation** H: H for **Kruskal Wallis test,** pairwise comparison between each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

P: P value for comparing between the three studied groups

P_1: P value for comparing between **Alone** and **AZT**

P_2: P value for comparing between **Alone** and **FO**

P_3: P value for comparing between **AZT** and **FO**

*: Statistically significant at P≤ 0.05

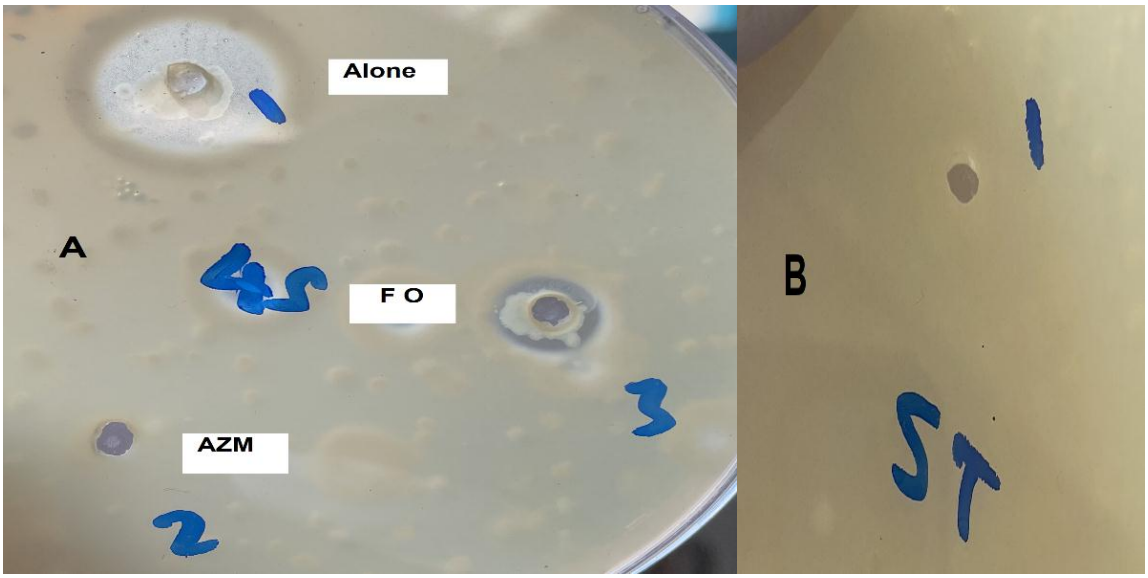

Fig. 1. Protease activity [A: Skimmed milk agar plate showing protease activity in the form of a halozone for *S. maltophilia* isolate in the absence or presence of AZM (complete loss of the halo zone) or FO (decrease in the halo zone diameter) AZM: Azithromycin (1/2 MIC), FO: Fenugreek oil (1/2 MIC), B: Absent protease activity in *S. maltophilia* ATCC 13637]

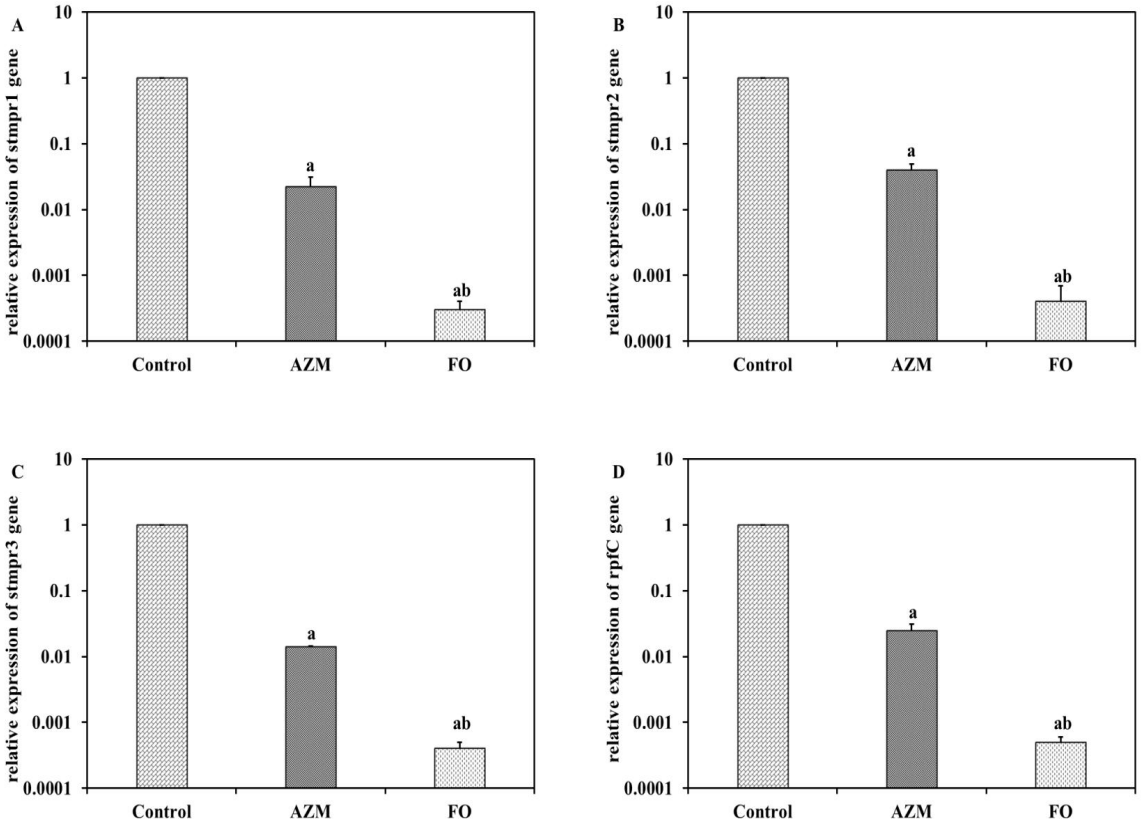

Fig. 2. Downregulation of some virulence genes of *S. maltophilia* isolates by each AZM and FO. ½ MIC of each AZM and FO produced a significant reduction in the expression levels of all investigated genes [A *stmpr1* gene, B *stmpr2* gene, C s*tmpr3* gene, D *rpfC* gene. The data shown represent the means ± standard deviations. One-way ANOVA was performed. Pairwise comparisons between 2 groups was done using Post Hoc Test (Tukey), with statistical significance at P≤ 0.05. a: Significant comparedwith the control group (untreated), b: Significant compared with the AZM group]

In this study, 21 out of the 50 *S. maltophilia* clinical isolates (42%) were MDR and were included in further assays. In an Egyptian study, Daef et al revealed that 63.5% of *S. maltophilia* isolates were MDR. (Daef et al., 2017). Additionally, Saleh et al reported 91% MDR strains in their study that was performed in Iraq (Saleh et al., 2021). Conversely, in an American study, Appaneal et al presented 3.1% MDR isolates and stated the stability of this percentage throughout their study period from 2010 to 2018. (Appaneal et al., 2022).

Concerning biofilm formation, out of the 21 isolates, 4 (19.05%) isolates strongly produced biofilms, and 17 (80.95%) isolates moderately produced biofilms. AZM showed a statistically significant difference in biofilm formation (P<0.001), with a mean percentage inhibition of 49.7%. In contrast, FO did not show a significant statistical relation but still showed a mean percentage inhibition of 18.5%. The *S. maltophilia* ATCC 13637 strain was a moderate biofilm producer that changed to a weak producer in the presence of AZM (73.3% inhibition) and remained a moderate producer with FO but with 39.8% inhibition.

Previous works studied biofilm formation in *S. maltophilia*. Azimi et al found that 98.7% of the studied isolates formed biofilms, where 46% formed strong biofilms, 21.3% formed moderate biofilms and 31.3% formed weak biofilms (Azimi et al., 2020). Saleh et al reported that 50% of the strains produced strong biofilms, of which 82% were MDR (Saleh et al., 2021). Wang et al reported the percentage of biofilm formation among their studied isolates as 77.8% strong, 15.6% moderate, 4.4% weak and 2.2% nonproducers, and they also proved a significant inhibitory effect for biofilm formation for AZM (Wang et al., 2016).

Regarding motility, there was a significant decrease in the interstitial zone surface area in the presence of either agent. As all the isolates (100%) had twitching motilities, but in presence of each of AZM and FO, a decrease in the surface area of the interstitial zones was observed by a mean value of 34.4% and 35.5%, respectively. *S. maltophilia* ATCC 13637 showed a complete inhibition of twitching motility with AZM, but this was not the same as FO, which only caused a 19% decrease in the interstitial zone surface area. Kalidasan and Neela investigated twitching motility in *S. maltophilia* and found that 67.85% (19/28) of the isolates had twitching motility (Kalidasan & Neela, 2020), while Cruz-Córdova et al. stated that 43.33% (13/30) of the isolates only showed twitching motility (Cruz-Córdova et al., 2020).

Protease activity was detected in all the tested isolates but was absent in the *S. maltophilia* ATCC 13637 strain. Interestingly, AZM showed complete inhibition of protease activity in all the tested isolates, while FO showed significantly decreased protease activity (decreased halo zone). Strateva et al. (2023) observed protease activity phenotypically in 95% of their isolates. On the other hand, Kim et al. (2018) detected protease activity for the tested clinical isolate using casein agar, and unlike the findings of this study, protease activity was detected for the *S. maltophilia* ATCC 13637 strain. This dissimilarity in the strain proteolytic activity may be related to the difference in the substrates and media used (Travassos et al., 2004).

The phenotypic inhibitory effect of each AZM and FO on the investigated virulence factors was confirmed using qRT–PCR for 3 protease genes and a QS-related gene. ½ MIC of either agent showed a significant downregulation in the 3 investigated protease genes (*stmpr1*, *stmpr2* and *stmpr3*) and the QS-related gene (*rpfC)* compared with the untreated isolates. This strongly agrees with the phenotypic test results. Since DSF-QS in *S. maltophilia* is an important factor for regulating virulence-associated genes and biofilm formation, therefore interfering with DSF signals will lead to loss of motility, impaired protease activity and decreased biofilm formation (Trifonova & Strateva, 2019).

Meanwhile, the standard strain *S. maltophilia* ATCC 13637 showed a higher relative expression level in the presence of each of AZM and FO for the protease genes but showed a lower relative expression level of the *rpfC* QS-related gene with AZM (14%) and with FO (10%). This downregulation of QS-related gene expression can explain the corresponding decreased phenotypic characteristics, including motility and biofilm formation, for the strain. In contrast, protease gene expression upregulation in the presence of AZM or FO provides a discrepancy with the absence of phenotypic protease activity in the absence of either agent. Upregulated gene expression that corresponds to absent phenotypic characteristics may be explained through phenotypic heterogeneity phenomenon; due to different genetic or epigenetic factors (Darja, 2007; Magdanova & Golyasnaya, 2013; Chowdhury et al., 2021). Another probable explanation for this discrepancy is the presence of macromolecular crowding and aggregation of mRNAs and proteins forming complexes that are immobile or slowly mobile, contributing to slow diffusion and localization of mRNAs and hence greatly affecting translation (Zhdanov, 2014).

AZM was reported for its anti-virulence effects by other studies (Imperi et al., 2014; Aleanizy et al., 2021; Elsheredy et al., 2021; Thomsen et al., 2021). Like *S. maltophilia*, *Pseudomonas aeruginosa* is a gram-negative bacterium that is naturally resistant to AZM and has no approved breakpoints. These studies reported that sub-MIC of AZM against *Pseudomonas aeruginosa* had an antibiofilm effect and decreased the production of many virulence factors, including motility, protease activity and pyocyanin production. These effects were related mainly to the anti-QS effect of AZM.

Although the exact mechanism of AZM as an anti-QS drug is not exactly understood, evidence has indicated that AZM (as a macrolide) affects QS signals by inhibiting ribosomes. Hence, AZM at sub-MICs causes selective translational inhibition and thus affects the expression of QS-dependent genes (Imperi et al., 2014; Baldelli et al., 2020). This may also provide an explanation for the results presented in this study, where AZM sub-MIC phenotypically affected the biofilm formation, protease activity and motility of *S. maltophilia* isolates. The relative genes expression results also showed that AZM caused downregulation of protease genes and the QS-related gene (*rpf*C*)*. Nevertheless, more research studies are required to emphasize the exact mechanism of AZM against *S. maltophilia* QS and virulence factors.

Some studies revealed that some spice essential oils and/or one of their components had an inhibitory effect on QS systems either through inhibiting QS signals expression or their corresponding receptors. Consequently, this can affect QS-dependent virulence factors (Chang et al., 2014; Myszka et al., 2016; Zhang et al., 2020). Moreover, they can affect motility through downregulation of genes regulating flagellin production or flagellar assembly (Kollanoor-Johny et al., 2012).

In addition, some studies have proven that some spice essential oils and their bioactive compounds can affect biofilm formation by interfering with the initial adherence step of planktonic cells (Silva et al., 2016), downregulating fimbriae gene expression or even reducing extracellular polymeric substance production (Kim et al., 2016; Zhang et al., 2020). Nevertheless, the exact effect of FO on virulence factors in *S. maltophila* or any other bacteria was not explained before, so further studies are needed to explain the anti-virulence effects detected in this study and highlight the main components of the oil that provided these effects and the proposed mechanisms.

This study had the following limitations. The study focused on a limited number of virulence factors and did not include other important factors, such as siderophores, lipopolysaccharides and other extracellular enzymes, such as DNase, esterases, lipases, hyaluronidase and alkaline phosphatases. The genotypic assay was conducted only on a small number of isolates due to limited financial resources. No clinical studies have been conducted on animal models to evaluate the inhibitory effect in vivo. Nevertheless, the provided results confirm a significant potential anti-virulence effect against MDR clinical isolates of *S. maltophila*.

Conclusion

S. maltophilia is a nosocomial opportunistic bacterium of great global clinical concern as an MDR pathogen. AZM and FO demonstrated antibiofilm and anti-virulence effects against MDR *S. maltophila* isolates. AZM showed significant inhibitory effects against protease activity (100% inhibition), followed by motility and biofilm formation. Meanwhile, FO had a significant effect against protease activity and motility but did not show a significant difference in biofilm formation, still showing a mean percentage inhibition of 18.5%. At the level of gene expression (protease genes and *rpf*C QS-related gene), both agents also showed significant downregulation. However, determination of their exact molecular mechanisms as anti-QS and anti-virulence agents is highly recommended.

List of abbreviations: Azithromycin (AZM), Bronchoalveolar lavage (BAL), Diffusible signal factor (DSF), Fenugreek oil (FO), Minimum inhibitory concentration (MIC), Multidrug-resistant (MDR), Optical density (OD), Quorum sensing (QS) *Stenotrophomonas maltophilia* (*S. maltophilia*).

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Ethics approval: The research design has been approved in 2021 by the Unit of Research Ethics Approval Committee (UREAC) of Pharos University in Alexandria under the approval number of 01/2021/03/16/3/001. The research was performed on bacterial isolates collected from clinical samples that were already cultured as part of the routine work in the microbiology laboratory of three different hospitals in Alexandria, Egypt. No human participants, their data nor biological material from them was utilized in the research.

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تقييم تأثير الأزيثرومايسين و زيت الحلبة كعوامل مضادة لضراوة بكتريا الستينوتروفوموناس مالتوفيليا من العزلات السريرية المتعددة المقاومة للمضادات الحيوية

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يعتبر ستينوتروفوموناس مالتوفيليا أحد البكتيريا الانتهازية المتسببة في الأمراض والمرتبطة بعدوى المستشفيات و يعتبر أحد أهم الميكروبات المتعددة المقاومة للمضادات الحيوية.

يُعرف ''أزيثرومايسين'' (مضاد حيوي -مجموعة الماكروليد) بتأثيراته المضادة لعوامل الضراوة البكتيرية و تأثيره كمضاد لاستشعار النصاب بين الخلايا وأيضاً، عُرِفت بعض زيوت التوابل العطرية بتأثيرها كمثبطات لعوامل الضراوة البكتيرية. وقد قامت هذه الدراسة بتقييم تأثير كلاً من أزيثرومايسين وزيت الحلبة (كزيت توابل عطري) ضد بعض عوامل الضراوة البكتيرية لعزلات ''ستينوتروفوموناس مالتوفيليا'' متعددة المقاومة للمضات لحيوية. وقد تم تقييم تأثير كلاً منهما بشكل ظاهري وعلى مستوى التعبير الجيني لبعض الجينات المتعلقة بعوامل الضراوة، مما يرجح استخدامهما كعلاج مضاد لعوامل الضراوة في حالات العدوي الناتجة عن ''ستينوتروفوموناس مالتوفيليا'' المتعددة المقاومة للمضادات الحيوية.

وقد أظهر ''أزيثرومايسين'' تأثيرات تثبيطية ملحوظة ضد إنتاج البروتياز (100% تثبيط)، تليها الحركة البكتيرية (انخفاض 34.4%) وتكوين البايوفيلم البكتيري (تثبيط 49.7%). وكذلك، كان لزيت الحلبة تأثير كبير ضد إنتاج البروتياز (انخفاض بنسبة 25.0 - 53.3%) والحركة البكتيرية (انخفاض 35.5%) و على الرغم من عدم وجود علاقة إحصائية في تكوين البيوفيلم البكتيري، إلا أنه وجدت نسبة تثبيط متوسطها 18.5%. أما بالنسبة للتعبير الجيني (جينات البروتياز والجين المتعلق باستشعار النصاب بين الخلايا (rpfC)، أثبت كلا من ''الأزيثرومايسين'' و زيت الحلبة تنظيمًا سلبيًا في التعبيير الجيني.