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Effects of erythromycin, nystatin and their combination on *Pseudomonas aeruginosa* and *Candida albicans* biofilms

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ABSTRACT:

Introduction: *Pseudomonas aeruginosa* is frequently found to coexist and interact with *Candida albicans* in the polymicrobial biofilms. Considering the pathogenicity of polymicrobial biofilms in many persistent infections, this study was aimed to evaluate the combined effects of antibiotics erythromycin and nystatin on *P. aeruginosa-C. albicans* polymicrobial biofilm under both aerobic and anaerobic conditions.

Methods and Results: Treatment with single (erythromycin or nystatin) and combined antibiotics caused subtle reductions in the number of viable biofilm cells as well as protein and extracellular DNA content of the biofilm extracellular polymeric substance (EPS). Protein profiling demonstrated differential expression of biofilm proteins following exposure to the antimicrobial treatments.

Conclusion: These findings suggest that erythromycin and nystatin, either single or in combination affect EPS composition (protein and extracellular DNA) as well as biofilm protein expression.

Keywords: Biofilm, *Pseudomonas aeruginosa*, *Candida albicans*, erythromycin, nystatin

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1. Introduction

The polymicrobial infection in which microorganisms interact synergistically severly endangers health. Microorganisms rarely exist as single-species biofilms but instead tend to develop complex polymicrobial biofilm communities attached to biotic and abiotic sites. Biofilm cells are well protected by the hydrated extracellular polymeric substance (EPS) matrix [1]. The polymicrobial biofilm has been shown to be more resistant to biocides than monomicrobial biofilm [2]. The relationship between the microbial species relies on contact-dependent attachment and quorum-sensing system [3]. This shows that the tendency of various microbial species to coexist is attributed to how well they communicate with each other.

Combined antibiotics are designed to improve therapy efficacy through synergistic action and to overcome resistance in polymicrobial infections. There have been increasing efforts to develop combined antibiotics aiming to fight against the difficult-to-treat infections. For instance, clinical studies show that augmentin (an antibiotic formulation of amoxycillin trihydrate and potassium clavulanate) to be effective against causative organisms that are resistant to amoxycillin alone but sensitive in the presence of potassium clavulanate [4]. In addition, reduction in the viability of *C. albicans* by amphotericin B in polymicrobial biofilm which then allows *S. aureus* to respond to vancomycin has also been reported [5]. Hence, combined antibiotics have been regarded as the last line of defense in combating the prevalent biofilm-mediated infections .

A common example of polymicrobial biofilm is the coaggregation between *Pseudomonas aeruginosa* and *Candida albicans*. This polymicrobial biofilm has frequently been found in ventilator-associated infections, as well as pneumonia and cystic fibrosis-related infections [6,7]. The antagonistic interaction between bacteria and fungi has been observed in vitro whereby *P. aeruginosa* forms a dense biofilm on *C. albicans*

filaments and kills the fungus [8]. This inhibits *C. albicans* filamentation from growing resistant yeast form [9]. Thus the biofilm formation by *P. aeruginosa* causing pneumonia in rats is believed to be a result of precolonization of lung tissue by *C. albicans* [10]. Collectively, a clear picture of how they coexist is well established. However, very few investigations on using combined antibiotics to combat this polymicrobial biofilm have been reported.

The understanding of the molecular mechanism of a potential antimicrobial substance may help ensuring its suitable application in controlling infections. It is generally accepted that the molecular mechanisms underlying the antimicrobial effects usually involve cell membrane modification, changes in ion flux and alteration of protein expression [11]. Regulating protein expression and the metabolic activities by the antimicrobial substances can adversely affect the survival of the target microorganism [12,13]. Therefore, it is possible that the antibiofilm mechanism of combined antibiotics is also mediated by changes in protein expression. This work was conducted to evaluate the combined effects of erythromycin and nystatin on *P. aeruginosa*-*C. albicans* polymicrobial biofilms under aerobic and anaerobic conditions.

2. Materials *&* Methods

2.1. Preparation of antibiotics

Erythromycin and nystatin were dissolved in 95 % ethanol and distilled water respectively, which were then filtered through 0.45 µm Sartorius filters. The single antibiotics (erythromycin or nystatin) and combined antibiotics (in a ratio of 1:1) were prepared in five different concentrations as follows: 40 mg ml^{-1}, 20 mg ml^{-1}, 10 mg ml^{-1}, 5 mg ml^{-1} and 2.5 mg ml^{-1}.

2.2. Preparation of test microorganisms

C. albicans strain ATCC 10231 was grown in potato dextrose broth (PDB) at room temperature in the absence of light while *P. aeruginosa* strain ATCC 10145 was grown in Mueller Hinton broth (MHB) at 35 °C. The microbial growth patterns were monitored by CFU counting.

2.3. Biofilm Assay

P. aeruginosa and *C. albicans* were grown in the cultures until they reached microbial density equivalent to 10^4 CFU mL^{-1} (6 and 22 hours respectively). For monobiofilm preparation, a volume of 5 ml of each microbial culture was transferred into 6-well plate and incubated at 37 °C for 24 hours. Meanwhile, for polybiofilm preparation, a volume of 2.5 ml from microbial cultures of *P. aeruginosa* and *C. albicans* were transferred into a 6-well plate resulting in a 1:1 ratio of *P. aeruginosa* and *C. albicans* suspension with a total volume of 5 ml in each well (5). The plate was then incubated for 24 hours at 37 °C. The monobiofilm and polybiofilm culture plates were prepared twice for two different environmental conditions, aerobic and anaerobic.

2.4. Antibiofilm treatment

At 24 hours post incubation, the media containing planktonic suspensions was discarded and the wells containing biofilm cells were washed once with phosphate buffer saline (PBS) pH 7.4. The test wells containing monobiofilm and polybiofilm cells were then added with fresh broth (4 mL) and antibiotics solution (2 mL). The control well contained only 6 mL of fresh broth. The plate was incubated for 24 hours at 37 °C.

2.5. Preparation of biofilm suspension

After 24 hour incubation, biofilm cells were suspended in PBS buffer and physically detached from the wells. The biofilm suspension was then used for: i) CFU counting, ii) biochemical composition analysis of EPS and iii) protein electrophoresis.

2.6. CFU counting

The CFU counting was performed based on (5) with some modifications. The biofilm suspension collected from the microtiter plate biofilm formation assay was diluted to a ratio of $1:10^6$ using fresh broth. A volume of 10 µL of the biofilm suspension was streaked onto sterile Mueller Hinton agar (*P. aeruginosa* biofilm), Potato Dextrose agar (*C. albicans* biofilm) and Brain Heart Infusion agar (*P. aeruginosa*-*C. albicans* polymicrobial biofilm). The plates were then incubated for 24 hours at 37 °C. The viable cells were counted the following day to obtain CFU mL^{-1} value. The CFU counting was performed in triplicates.

2.7. Determination of biochemical composition of EPS matrix

The EPS extraction method was conducted based on (14) with minor modifications. The biofilm suspension from the 6-well plate was pelleted by centrifugation (5000 rpm, 30 mins, 4 °C) and resuspended in EPS extracting solution (100 mM NaCl, pH 7.0). The cells were pelleted by centrifugation and resuspended again in the EPS extracting solution. The EPS components were extracted by shaking for 1 hour at 30 °C in a water bath at 100 rpm, and the supernatant solution was recovered by centrifugation (5000 rpm, 30 mins, 4 °C). The supernatant solution was then centrifuged (12000 rpm, 30 mins, 4 °C) to remove residual cells. A volume of 200 µl of EPS extract was precipitated by adding 600 µl of

cold absolute ethanol, and the suspension was stored at -20 °C for 18 hours. The EPS crude extract was separated from the supernatant solution by centrifugation (12000 rpm, 30 mins, 4 °C). The amount of protein in crude EPS extract was measured using Bradford assay at 595 nm whereas the amount of nucleic acid was measured at 260 nm. All the measurements were performed in triplicates.

2.8. Protein electrophoresis

The biofilm suspension from the 6-well plate was pelleted by centrifugation at 8000 rpm for 10 minutes which was then suspended in ice-cold RIPA lysis buffer (0.5 M Tris-HCL, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA and 5% protease inhibitor) and mixed well by vortex. The cell lysate was centrifuged at 13000 rpm for 10 minutes to obtain the clear supernatant. 12% SDS polyacrylamide gel electrophoresis was performed according to NuPAGE® electrophoresis system at 200 V for 35 minutes. The polyacrylamide gels were then heated in a microwave for one minute, incubated with Coomassie Brilliant Blue stain for three hours and destained overnight in distilled water. The gels were photographed and analyzed using Alpha Imager and GS-800 USB Calibrated Densitometer respectively. Experiments were performed in triplicates.

2.9. Densitometric analysis

The relative intensity of electrophoretically separated protein bands was determined using GS-800 USB Calibrated Densitometer in the range between 400 nm and 750 nm. The gel images were imported into the Quantity One™ image analysis software and image contrast was adjusted to ensure protein bands were clearly visible. Then, the area of each protein band was selected and background intensity was subtracted from the gel image.

3. Results

3.1. Viable Bacterial and Fungal Biofilm Cells

In this study, the single and combined antibiotics were expected to reduce the number of viable biofilm cells. Although there have been several issues on the resistance of *P. aeruginosa* and *C. albicans* towards erythromycin and nystatin respectively, their combined antimicrobial potential at higher concentrations were still of interest. Thus, considering the survival of these microorganisms in the presence and absence of oxygen, the antibiotics were tested in the range between 2.5 mg mL^{-1} and 40 mg mL^{-1} under both aerobic and anaerobic conditions. In the case of combined antibiotics, the erythromycin was mixed with nystatin in a ratio of 1:1. As summarized in Table 1, both single and combined antibiotics were found to be effective against the biofilms under aerobic and anaerobic conditions. *P. aeruginosa* biofilm responded in a dose-dependent manner from 0 mg mL^{-1} to 20 mg mL^{-1} of erythromycin. The single antimicrobial assay of erythromycin 20 mg mL^{-1} resulted in a 98% decrease in viable biofilm cells under aerobic condition and 91.6% decrease under anaerobic condition as compared to the untreated control. *C. albicans* biofilm was affected by nystatin and the number of CFU decreased as the dose of the drug increased up to 10 mg mL^{-1}. The treatment with nystatin 40 mg mL^{-1} was found to completely inhibit the survival of *C. albicans* biofilm in culture under both aerobic and anaerobic conditions. *P. aeruginosa*-*C. albicans* biofilm viability was inhibited by combined antibiotics and the number of viable cells reduced as drug concentration increased in both conditions. Nevertheless, there were a slight increase about 1.13 % (from 5 to 10 mg mL^{-1} combined antibiotics) and 0.13 % (from 2.5 to 5 mg mL^{-1} combined antibiotics) under aerobic and anaerobic conditions respectively. The combined antimicrobial assay demonstrated that 40 mg mL^{-1} combined antibiotics exhibited the strongest killing effect against the polymicrobial biofilm with 97.7% and 95.6% reductions in viable cell count under aerobic and anaerobic environments respectively.

3.2. Comparative Composition of Monomicrobial and Polymicrobial Biofilm EPS

The protein content extracted from biofilm EPS was determined using spectrophotometry at 595 nm, whilst the extracellular DNA content of biofilm EPS was measured at 260 nm. As listed in Table 2, the single antimicrobial assay demonstrated that only 40 mg mL^{-1} erythromycin apparently reduced protein content of *P. aeruginosa* biofilm EPS under aerobic condition whilst both 20 mg mL^{-1} and 40 mg mL^{-1} of erythromycin were found to apparently decreased protein content of *P. aeruginosa* biofilm EPS under anaerobic condition. On the other hand, under aerobic condition, only nystatin 40 mg mL^{-1} reduced protein content of *C. albicans* biofilm EPS while under anaerobic condition, all the test concentrations of nystatin were observed to reduce protein content of *C. albicans* biofilm EPS. Meanwhile, the treatment with combined antibiotics resulted in lower protein content of polymicrobial biofilm EPS at all the test concentrations as compared to the control under aerobic condition whereas test concentrations of 10 mg mL^{-1} and above of combined antibiotics caused reduction in the protein content of polymicrobial biofilm EPS under anaerobic condition.

Based on Table 3, the exposure of *P. aeruginosa* biofilm to the test concentrations of erythromycin ranged between 10 - 40 mg mL^{-1} caused a reduction in the extracellular DNA content *P. aeruginosa* biofilm EPS under aerobic condition whilst all the test concentrations were found to decrease extracellular DNA content of *P. aeruginosa* biofilm EPS under anaerobic condition. Unfortunately, only nystatin 40 mg mL^{-1} was shown to reduce extracellular DNA content of *C. albicans* biofilm EPS under aerobic condition and no decrease in extracellular DNA content of *C. albicans* biofilm EPS was observed under anaerobic condition. Noticeably, the treatment with 5 to 40 mg mL^{-1} of combined antibiotics produced a large decrease in the extracellular DNA content of polymicrobial biofilm EPS under aerobic condition. However, the extracellular DNA content of polymicrobial biofilm EPS was not substantially affected by the treatment with 10 to 40 mg mL^{-1} of combined antibiotics under anaerobic condition.

3.3. Differential Biofilm Protein Expression

The test concentrations of combined antibiotics (40 mg mL^{-1}) and single antibiotic (40 mg mL^{-1}) were used in the protein profiling based on their efficiency in reducing the biofilm cell viability, as well as EPS protein and DNA contents. Figure 1 presents the differential protein expression under aerobic and anaerobic conditions. In contrast, there were no apparent changes observed in the protein profile of *P. aeruginosa* biofilm following the treatment with erythromycin 40 mg mL^{-1} (Figure 1a). Meanwhile, the treatment with nystatin 40 mg mL^{-1} caused profound changes in the total protein profile of *C. albicans* biofilm as compared to the control (Figure 1b). The treatment with 40 mg mL^{-1} combined antibiotics resulted in substantial changes in the total protein profile between control and treated polymicrobial biofilm (Figure 1c).

The polyacrylamide gels were subjected to densitometry analysis for determination of approximate relative concentrations of biofilm total proteins. The high sensitivity of densitometry analysis enables the identification of even poorly stained protein bands.

Table 4 (top panel) shows the optical density of total proteins of *P. aeruginosa* biofilm following exposure to 40 mg mL^{-1} erythromycin. There were 10 proteins exhibiting differential expression upon treatment. The fold change values were found to range between 1.37 and 2.34. Except for the protein of 14 kDa, the fold change values of all biofilm proteins under anaerobic condition were lesser compared to those under aerobic condition. Table 4 (middle panel) depicts optical density of total proteins of *C. albicans* biofilm upon exposure to 40 mg mL^{-1} nystatin. Noticeably, the fold change values ranged from 2.0 to 5.27. Furthermore, the differential expression of proteins of 55 kDa, 47 kDa and 21 kDa was greater under aerobic condition. As listed in Table 4 (bottom panel), a total of 10 proteins from the polymicrobial biofilm were found to be downregulated upon the treatment with the 40 mg mL^{-1} combined antibiotics under aerobic and anaerobic conditions. The fold change values ranged from 3.38 to 9.16. It was also noted that the fold change values of all biofilm proteins under anaerobic condition were greater than those under aerobic condition.

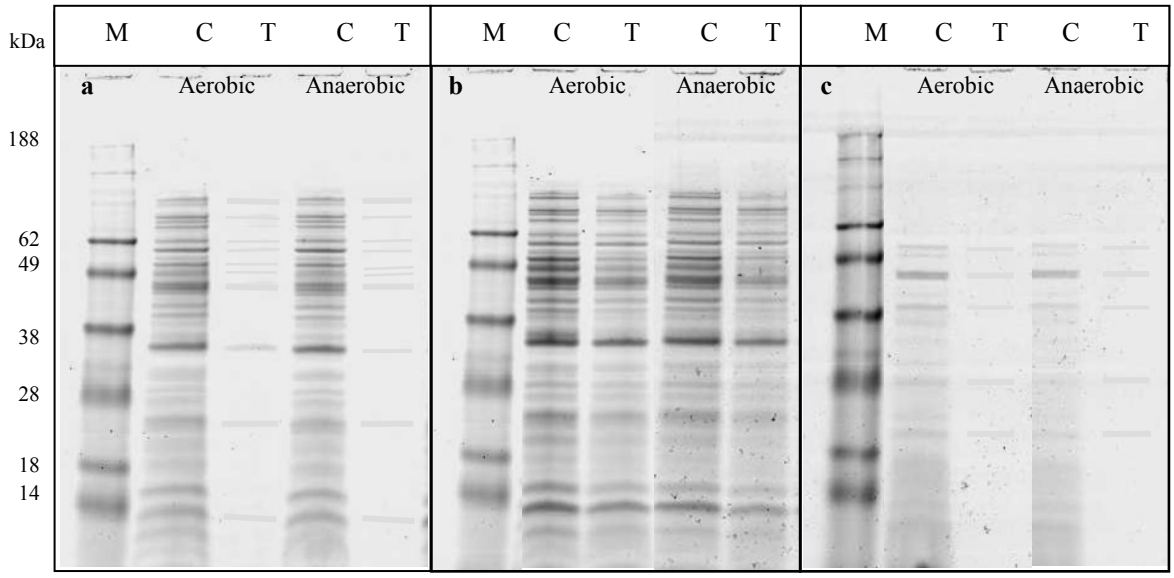

Figure 1. Biofilm protein profiles under aerobic and anaerobic conditions. **a** *P. aeruginosa* monomicrobial biofilm, **b** *C. albicans* monomicrobial biofilm, and **c** *P. aeruginosa*-*C. albicans* polymicrobial biofilm upon treatment with the single erythromycin (40 mg ml^{-1}), single nystatin (40 mg ml^{-1}) and combined antibiotics (40 mg ml^{-1}) respectively. **M** protein marker, **C** control group, and **T** test group.

4. Discussion and Conclusion

Both *P. aeruginosa* and *C. albicans* are able to establish biofilms under aerobic and anaerobic conditions. In order to investigate the antibiofilm activity of single and combined antibiotics, aerobic and anaerobic conditions should be considered as oxygen limitation has been reported to contribute to the antimicrobial tolerance in biofilm infections (15). Several preliminary studies have previously been performed to understand the effects of oxygen supply in antimicrobial activities. A group of researchers found that the tissue oxygenation and antibiotic administration had an additive effect on decreasing infectious necrosis (16). Meanwhile, it was earlier reported that the antibiotics which reduced anaerobic bacterial population in gut made animals vulnerable to *C. albicans* mucosal association (17). Furthermore, differential susceptibility of *P. aeruginosa* biofilm towards common medicinal plant extracts under aerobic and anaerobic conditions has also been reported (18,19). Together with those findings, the CFU count data following antibiotic treatments suggest that the effects of single and combined antibiotics upon monomicrobial and polymicrobial biofilms respectively may vary under different oxygen levels.

Proteins are one of the important biopolymers that determine the architecture and morphology of the biofilm matrix. The critical function of protein in EPS is to facilitate the microbial attachment to different solid surfaces with its tertiary structure, charged groups and electrostatic forces. Therefore, the reduction of protein content of EPS is vital to control the biofilm development. Treatment with ciprofloxacin was reported to cause a change in the bacterial infrared spectrum which corresponded to proteins as a result of reduction in CFU count of the biofilm cells (20). It was then shown a decrease in the level of biofilm protein by erythromycin at the concentrations of 10 $\mu g\ mL^{-1}$ and higher was dose-dependent (21). Recently, the similar finding has been demonstrated by other investigations using antimicrobial agent dimethyl sulfoxide (22,23). Taken together, these studies (20-23) support our results whereby the treatments with the single and combined antibiotics could decrease the protein content of biofilm EPS.

DNA is secreted into the extracellular space upon biofilm formation in order to bind with other biopolymers and provide structural integrity to EPS. The extracellular DNA is released into the biofilm EPS matrix and is required to establish the initial attachment and biofilm structure of *Pseudomonas* species (24). Treatment by DNAse was proposed to cause a destruction of extracellular DNA and enabled the increased penetration of antibiotics (25). The study was supported by another study that showed the presence of extracellular DNA in *C. albicans* biofilm EPS and its susceptibility to the DNAse treatment (26). The findings herein also have indicated the reduction in extracellular DNA content of biofilm EPS under aerobic and anaerobic conditions following the exposure to single and combined antibiotics. The observed antibiofilm activity may be mediated by the damage of extracellular DNA which permits destabilization of the biofilm architecture and enhanced penetration of antibiotics. Thus, it becomes clear that targeting extracellular DNA is a promising strategy for the biofilm control.

The effect of antibiotics upon organism's protein can be visualized through SDS-PAGE in combination with protein identification method by mass spectrometry. The analysis of protein expression of biofilm cells could provide an insight into how the antibiotics could control the infectious agent. For instance, treatments with clindamycin and linezolid antibiotics resulted in suppression of Panton-Valentine Leukocidin proteins in Methicillin-resistant *Staphylococcus aureus* (MRSA)(27). Besides that, a study that has employed the method of protein profiling was able to show that a total of 302 proteins were upregulated upon the treatments of tetracycline and imipenem against *Acinetobacter baumannii* which included ribosomal proteins, iron-uptake transporters and β-lactamase (28). The protein profiling in this study has shown the influence of single and combined antibiotics on the total proteins of monomicrobial and polymicrobial biofilms under aerobic and anaerobic conditions. To investigate the identity of differentially expressed proteins in the monomicrobial and polymicrobial biofilms, our future work will involve electrophoretic separation of biofilm proteins using the two dimensional gel electrophoresis and mass spectrometry. The results will lead to the identification of proteins and their functions to explain the molecular mode of action of single and combined antibiotics against monobiofilm and polybiofilm.

The treatments with single and combined antibiotics showed reduction in protein and extracellular DNA contents of biofilm EPS which may facilitate the killing of *P. aeruginosa* and *C. albicans* biofilms. The treatments also caused reduction in the biofilm total proteins which may enhance the biofilm susceptibility towards antibiotics. The combined antibiotics strategy may promise an effective strategy in combating the polymicrobial biofilm.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Table 1. Viability of biofilms upon antibiotics treatment. *P. aeruginosa, C. albicans* and *P. aeruginosa-C. albicans* biofilm cells treated with erythromycin, nystatin and erythromycin:nystatin respectively under aerobic and anaerobic conditions. Data represent averages of triplicates.

Antibiotic concentrations (mg mL^{-1})	Viable Cells (CFUs mL^{-1}) ± SD					
	P. aeruginosa		*C. albicans*		*P. aeruginosa-C. albicans*	
	Aerobic (10^7)	Anaerobic (10^7)	Aerobic (10^6)	Anaerobic (10^6)	Aerobic (10^7)	Anaerobic (10^7)
40	0.92 ± 0.27	1.13 ± 0.10	0	0	0.16 ± 0.08	0.33 ± 0.07
20	0.08 ± 0.02	0.32 ± 0.02	0.13 ± 0.12	0.20 ± 0.26	1.57 ± 0.10	0.77 ± 0.57
10	0.96 ± 0.19	1.07 ± 0.33	0.03 ± 0.06	0.10 ± 0.17	1.76 ± 0.47	1.08 ± 0.18
5	1.51 ± 0.22	1.74 ± 0.23	0.27 ± 0.21	0.40 ± 0.26	1.68 ± 0.22	1.73 ± 0.13
2.5	1.70 ± 0.15	1.94 ± 0.67	0.47 ± 0.21	0.40 ± 0.26	2.18 ± 0.54	1.72 ± 0.58
0 (Control)	4.08 ± 0.46	3.82 ± 0.90	2.77 ± 0.91	2.67 ± 0.32	7.07 ± 0.81	7.67 ± 0.88

Table 2: Protein content of biofilms EPS upon treatment. Data are expressed as mean ± standard deviation.

Antibiotic concentrations (mg mL^{-1})	Protein content (µg µL^{-1}) ± SD					
	P. aeruginosa		*C. albicans*		*P. aeruginosa-C. albicans*	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
40	0.0089 ± 0.00	0.0368 ± 0.01	0.0017 ± 0.00	0.0092 ± 0.01	0.0688 ± 0.05	0.0395 ± 0.02
20	0.0188 ± 0.01	0.1331 ± 0.01	0.0165 ± 0.01	0.0280 ± 0.00	0.0803 ± 0.05	0.0280 ± 0.02
10	0.0220 ± 0.00	0.1650 ± 0.01	0.0102 ± 0.01	0.0112 ± 0.01	0.0750 ± 0.04	0.0566 ± 0.02
5	0.0224 ± 0.01	0.1761 ± 0.02	0.0131 ± 0.01	0.0254 ± 0.01	0.0760 ± 0.04	0.1297 ± 0.03
2.5	0.1024 ± 0.01	0.1850 ± 0.02	0.0119 ± 0.01	0.0069 ± 0.00	0.2334 ± 0.03	0.2318 ± 0.07
0 (Control)	0.0204 ± 0.01	0.1771 ± 0.01	0.0131 ± 0.01	0.0583 ± 0.00	0.5712 ± 0.03	0.1185 ± 0.05

Table 3. DNA content of monomicrobial and polymicrobial biofilm EPS upon treatment. Data are expressed as mean ± standard deviation.

Antibiotic concentrations (mg mL^{-1})	DNA content (µg µL^{-1}) ± SD					
	P. aeruginosa		*C. albicans*		*P. aeruginosa-C. albicans*	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
40	0.007 ± 0.00	0.058 ± 0.00	0.031 ± 0.01	0.034 ± 0.00	0.016 ± 0.00	0.037 ± 0.00
20	0.019 ± 0.00	0.162 ± 0.00	0.055 ± 0.00	0.065 ± 0.00	0.016 ± 0.00	0.041 ± 0.00
10	0.040 ± 0.00	0.144 ± 0.00	0.086 ± 0.00	0.092 ± 0.00	0.040 ± 0.00	0.041 ± 0.00
5	0.050 ± 0.00	0.110 ± 0.00	0.078 ± 0.00	0.068 ± 0.00	0.119 ± 0.00	0.115 ± 0.00
2.5	0.134 ± 0.00	0.109 ± 0.00	0.071 ± 0.00	0.092 ± 0.00	0.352 ± 0.01	0.116 ± 0.00
0 (Control)	0.066 ± 0.00	0.232 ± 0.00	0.042 ± 0.01	0.032 ± 0.00	0.268 ± 0.00	0.058 ± 0.00

Table 4. Density of biofilms protein following antibiotics treatment. Protein density of *P. aeruginosa-C. albicans, P. aeruginosa* and *C. albicans* biofilm upon treatment with combined and single (erythromycin or nystatin) antibiotics respectively under aerobic and anaerobic conditions.

Molecular weight (kDa)	Aerobic		Fold change [Test/Control]	Anaerobic		Fold change [Test/Control]
P. aeruginosa	Control	Test		Control	Test	
117	+	+	-1.89	+	+	-1.45
91	+	+	-1.43	+	+	-1.17
62	+	+	-1.40	+	+	-1.27
59	+	+	-1.53	+	+	-1.38
53	+	+	-2.34	+	+	-1.90
47	+	+	-1.96	+	+	-1.73
43	+	+	-1.68	+	+	-1.55
36	+	+	-1.42	+	+	-1.35
25	+	+	-1.62	+	+	-1.31
14	+	+	-1.37	+	+	-1.38
Total	10	10		10	10	
Molecular weight (kDa)	**Aerobic**		**Fold change [Test/Control]**	**Anaerobic**		**Fold change [Test/Control]**
C. albicans	Control	Test		Control	Test	
55	+	+	-2.08	+	+	-2.00
47	+	+	-4.40	+	+	-4.16
41	+	+	-2.92	+	+	-2.92
29	+	+	-2.98	+	+	-3.17
21	+	+	-5.27	+	+	-2.75
Total	5	5		5	5	
Molecular weight (kDa)	**Aerobic**		**Fold change [Test/Control]**	**Anaerobic**		**Fold change [Test/Control]**
P. aeruginosa-C. albicans	Control	Test		Control	Test	
117	+	+	-7.15	+	+	-9.16
91	+	+	-3.74	+	+	-4.97
62	+	+	-4.41	+	+	-5.32
59	+	+	-3.99	+	+	-6.78
53	+	+	-7.02	+	+	-8.23
47	+	+	-5.29	+	+	-6.93
43	+	+	-5.87	+	+	-7.23
36	+	+	-3.38	+	+	-8.83
25	+	+	-3.88	+	+	-5.32
14	+	+	-3.85	+	+	-4.73
Total	10	10		10	10	

+ presence, - fold decrease