RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2022.020

ScienceAsia 48 (2022): **215–222**

Treatment with the combined antimicrobials triggers proteomic changes in *P. aeruginosa-C. albicans* polyspecies biofilms

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Received 11 Mar 2021
Accepted 21 Oct 2021

ABSTRACT: *Pseudomonas aeruginosa* is known to coexist and interact with *Candida albicans* in the polyspecies biofilms. In our previous work, treatment with the combined antimicrobials has been shown to significantly inhibit this polyspecies biofilm; however, the proteomic changes associated with this biofilm inhibition remain not well understood. Thus, the present work was performed to determine the proteome profile of *P. aeruginosa-C. albicans* polyspecies biofilms following treatment with the combined antimicrobials. The *P. aeruginosa-C. albicans* polyspecies biofilms were developed in 6-well microplates at 37 °C for 24 h under aerobic and anaerobic conditions. Following the treatment with 40 mg/ml combined antimicrobials (20 mg/ml erythromycin:20 mg/ml nystatin), whole-cell proteomes from the polyspecies biofilms were analyzed by a combination of two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE) and mass spectrometry. On the other hand, the differentially expressed proteins were analyzed using STRING database. Results demonstrated that 3 and 9 *P. aeruginosa* proteins were differentially expressed in treated polyspecies biofilms under aerobic and anaerobic conditions, respectively. Based on the protein interaction network, several biological pathways such as carbohydrate metabolism, protein metabolism, and secondary metabolite metabolism in *P. aeruginosa* might be affected by the treatment with combined antimicrobials. There were no differentially expressed *C. albicans* proteins identified herein. It is possible that the inhibition of the polyspecies biofilms by the combined antimicrobial is associated with multiple biological pathways. The combined antimicrobials used in the present study may be useful to manage the diseases caused by *P. aeruginosa-C. albicans* polyspecies biofilms.

KEYWORDS: *Pseudomonas aeruginosa*, *Candida albicans*, polymicrobial biofilm, erythromycin, nystatin, proteomics

INTRODUCTION

Biofilm is a heterogeneous microbial community that is well protected by the hydrated extracellular matrix [1, 2]. It is well regulated by signalling molecules and molecular expressions that respond to surrounding environments [3, 4]. A wide range of antibiofilm studies has been carried out to identify the potential substances that can inhibit the biomass, viability, and extracellular matrix of biofilms [5–7]. It has been established that microorganisms rarely exist as monospecies biofilms but tend to form complex polyspecies biofilms on biotic and abiotic surfaces. The tendency of various microbial species to coexist relies on contact-dependent attachment and quorum-sensing system [8]. For many years, the polyspecies biofilm is known to be more resistant to biocides than the monospecies biofilm [9]. Combined antibiotics substantially improve therapy efficacy through synergistic action and overcome resistance in polymicrobial infections. Due to that, there have been increasing efforts to develop combined antibiotics aiming to fight against the difficult-to-treat infections. There is evidence showing that amphotericin B inhibits *C. albicans* in the polyspecies biofilms which then increases the susceptibility of *Streptococcus aureus* to vancomycin [10].

P. aeruginosa-C. albicans polyspecies biofilms have frequently been found in ventilator-associated infections as well as pneumonia and cystic fibrosis-related infections [11, 12]. The antagonistic interaction between bacteria and fungi has been observed *in vitro* whereby *P. aeruginosa* kills the yeast and hyphae of *C. albicans* and forms biofilms on the killed hyphae [13]. In parallel with that, *P. aeruginosa* also inhibits *C. albicans* filamentation leading to the growth of resistant yeast form [14]. The biofilm formation by *P. aeruginosa* causing pneumonia is believed to be a result of precolonization of lung tissue by *C. albicans* [15]. Collectively, their coexistence is well understood. Nevertheless, very few investigations on the use of combined antimicrobials to combat this polyspecies biofilm have been reported.

Understanding the molecular mechanism of a potential antimicrobial substance may help to ensure its suitable application in disease control. 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 [16–18]. The regulation of protein expression and the metabolic activities by the antimicrobial substances can adversely affect the survival of the target microorganism [19, 20]. Therefore, it is

possible that the antibiofilm mechanism of combined antimicrobials is also mediated by the changes in protein expression.

Proteome is the complete set of all proteins expressed in living cells. It is sensitive to many environmental factors such as temperature, pressure, oxygen level, and drug treatment. A standard approach to study the proteome expression is a combination of polyacrylamide gel electrophoresis or liquid chromatography with mass spectrometry. In 2014, Trejo-Hernandez et al [21] compared the proteomes of *P. aeruginosa* and *C. albicans* in the mixed biofilms. They noticed that the interaction with *C. albicans* triggered proteomic changes in *P. aeruginosa* biofilm, and *C. albicans* biofilm also displayed altered proteome expression in response to *P. aeruginosa* biofilm. The proteomic aspects of single species biofilms of *P. aeruginosa* [22] and *C. albicans* [23] have also been characterized; however, the proteome expression in the *P. aeruginosa-C. albicans* polyspecies biofilms has not been extensively explored. Our previous work [24] demonstrated the efficacy of erythromycin in combination with nystatin against the polyspecies biofilms. Nonetheless, the proteomic changes associated with this biofilm inhibition remain not well understood. Thus, this work was performed to determine the effect of combined antimicrobials on proteome expression in *P. aeruginosa-C. albicans* polyspecies biofilms under aerobic and anaerobic conditions.

MATERIALS AND METHODS

Antimicrobials

Antimicrobials used herein were erythromycin and nystatin. They were dissolved in their solvents (95% ethanol–erythromycin; sterile distilled water–nystatin), diluted in distilled water, and filtered through 0.45 μm Sartorius filter. The combined antimicrobial agents were prepared in 1:1 ratio of erythromycin and nystatin. In this study, 40 mg/ml combined antimicrobials (20 mg/ml erythromycin:20 mg/ml nystatin) was used because it has been shown to be the most effective concentration against *P. aeruginosa-C. albicans* polyspecies biofilms [24].

Test microorganisms

P. aeruginosa strain ATCC 10145 was grown in Nutrient broth (NB) at 37 °C for 24 h. It was transferred and streaked on Nutrient agar (NA) plates and then incubated at 37 °C for 16 h. *C. albicans* strain ATCC 10231 from freezer stocks was transferred to Potato dextrose broth (PDB) and incubated at 37 °C for 24 h. The fungal culture was inoculated and streaked on Potato dextrose agar (PDA) plates, then incubated at 37 °C for 24 h. Isolated and pure colonies were picked for use on the next step.

Microplate biofilm assay

The polyspecies biofilms were developed in 6-well microplates with and without the presence of combined antimicrobials. 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. Following a 24 h incubation at 37 °C, nutrient medium was discarded, and the formed biofilm fractions were rinsed twice with distilled water, suspended in 0.9% sodium chloride (NaCl) solution (Sigma, USA), and pelleted by centrifugation at 10 000 rpm for 10 min. The microplate biofilm assay procedure was performed in 3 replicates under both aerobic and anaerobic conditions. The anaerobic condition was developed using candle jar and a strip of paper soaked in methylene blue dye.

Protein extraction and determination

The whole-cell proteome was extracted from the obtained pellet using a lysis buffer containing 0.5 M Tris-HCL, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, and 50 μl of protease inhibitor per millilitre lysis buffer. Cell disruption was improved by sonication on ice at 80% amplitude, 5 cycles with 45 s burst and incubated for 2 h on ice. The cell debris and intact cells were removed by centrifugation at 8000 rpm for 5 min. The supernatant containing protein fractions was stored at −20 °C until further use. The concentration of extracted protein was determined using the standard Bradford assay.

Two-dimensional polyacrylamide gel electrophoresis

Protein samples were initially precipitated using 2D-Clean Up Kit. Then, the protein samples were solubilized with rehydration buffer (7 M urea, 2 M thiourea, 2% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% immobilized pH gradient (IPG) buffer, and 0.002% (w/v) bromophenol blue) and left overnight to rehydrate into ZOOM IPG strips (7 cm immobilized pH gradient with non-linear pH gradient range 3–10). First dimension isoelectric focusing (IEF) was performed in the ZOOM IPGRunner Mini-Cell according to the following protocol: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 12 000 V for 1 h. The strips were subsequently equilibrated with equilibration buffer (1 × NuPAGE lithium dodecyl sulfate (LDS) Sample Buffer, NuPAGE Sample Reducing Agent, and 125 mM iodoacetamide (IAA)). Gel electrophoresis was performed using 10% gel at 200 V for 45 min. Gels were stained using SYPRO Ruby overnight. Protein spots were visualized using UV illuminator (Alpha Imager HP) at 610 nm. Two-dimensional proteome maps were analyzed to identify protein spots which differed significantly in intensity between control gels and test gels using Progenesis

proteomic analysis software. The volume of each spot was normalized against the total volume of all spots in the gel, and the normalized values were expressed as percentage spot volume. Spots with a fold-change of at least 1.0 were excised from the gels for in-gel digestion.

Trypsin digestion

The gel plugs were washed with 50 mM ammonium carbonate (NH_4CO_3) (5 min), followed by 70% acetonitrile (ACN) (15 min) and 100% ACN (5 min) at room temperature. The liquid was discarded following each washing step. The gel plugs were then dried using Speed Vac (2000 rpm, 4 °C, 15 min). Reduction and alkylation steps were performed by incubation with 10 mM dithiothreitol (DTT)/100 mM NH_4CO_3 for 30 min at 60 °C and with 55 mM IAA/100 mM ammonium bicarbonate (ABC) for 20 min in the dark at room temperature. Subsequently, the gel plugs were washed twice with 50% ACN/100 mM ABC (20 min each) and dehydrated with 100% ACN (15 min) at room temperature before overnight digestion in 25 µl of 7 ng/µl of trypsin. The digested peptides were pooled into a clean tube and dried using a vacuum centrifuge before MS analysis.

Mass spectrometry

Dried peptides were dissolved in 0.1% formic acid (FA) and desalted using ZipTip C18 (Millipore, Billerica, USA), according to the manufacturer's protocol. Next, the peptides were eluted with Elution solution (0.1% trifluoroacetic acid (TFA)/50% ACN) into a new microcentrifuge tube. Peptides were spotted on AnchorChip Standard Targets plate in duplicate. Matrix solubilization solution (α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN/0.1% TFA) was then spotted on each peptide spot and allowed to dry. Meanwhile, external calibrant spots solution was deposited onto calibrant anchor spots on the AnchorChip target and allowed to dry. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/TOF) was performed on an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Framingham, MA) whilst the resulting peptide mass spectra were submitted to the Mascot search engine (Matrix science) to search against the non-redundant database of the National Center for Biotechnology Information (NCBIprot).

Bioinformatics

Identified differentially expressed proteins were classified according to their known biological functions by database searching using SwissProt/TrEMBL. They were used to construct protein interaction network using STRING database (max interactions: 100; confidence level: high–0.7). The functional linkages predicted herein were based on neighbourhood, fusion-fission events, occurrence, text mining, and data imported from public databases of physical interactions.

RESULTS

Effect of oxygen level on proteome expression in polyspecies biofilms

Fig. 1 shows 2D proteome profiles of the polyspecies biofilms. Proteome profile of aerobic polyspecies biofilm was slightly different from that of anaerobic polyspecies biofilm. Approximately 123 and 157 protein spots were detected in the untreated polyspecies biofilms under aerobic and anaerobic conditions, respectively.

Effect of combined antimicrobials on proteome expression in aerobic polyspecies biofilm

There were 3 protein spots in the aerobic polyspecies biofilm showing differential expression following the treatment with the combined antimicrobials (Fig. 1). Table 1 shows identified biofilm proteins that undergo differential expression following the treatment with the combined antimicrobials. Expression of ATP synthase subunit beta, elongation factor Tu, and succinyl-CoA ligase subunit beta in *P. aeruginosa* biofilm was found to be downregulated with the fold change values of 1.15, 1.4, and 1.84, respectively.

Changes in proteome expression in anaerobic polyspecies biofilm following treatment with combined antimicrobials

There were 9 protein spots in the anaerobic polyspecies biofilm showing differential expression (Fig. 1). Expression of aconitate hydratase 2 and Succinyl-CoA ligase subunit beta in *P. aeruginosa* biofilm was found to be downregulated following the treatment with the combined antimicrobials with the fold change values of 1.36 and 3.14, respectively (Table 1). On the other hand, elongation factor G1, 30S ribosomal protein S1, ATP synthase subunit alpha, ATP synthase subunit beta, enolase, elongation factor Tu, and 4-hydroxyphenyl pyruvate dioxygenase in *P. aeruginosa* biofilm were found to be upregulated with the fold change values ranging between 1.24 and 5.65.

C. albicans biofilm proteins

P. aeruginosa biofilm culture was mixed with *C. albicans* biofilm culture to develop the polyspecies biofilms at 37 °C for 24 h. The whole-cell lysate of the polyspecies biofilm fraction was subjected to protein extraction and protein separation. There were no *C. albicans* biofilm proteins showing differential expression in the treated polyspecies biofilms under both aerobic and anaerobic conditions.

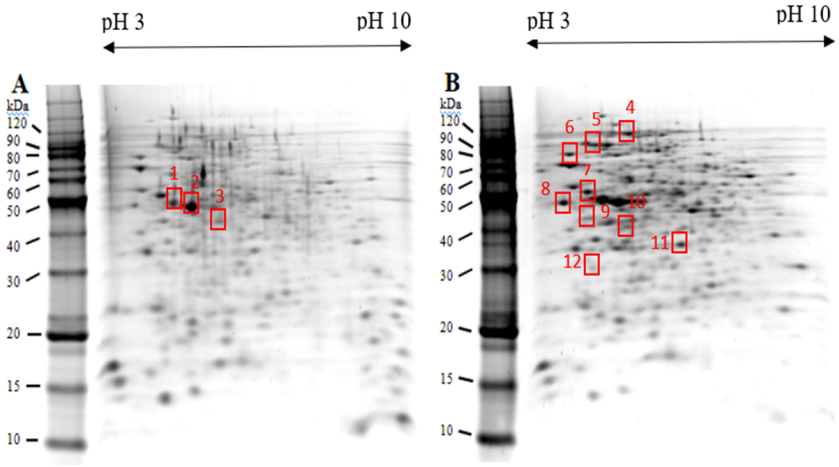


Fig. 1 Proteome maps of *P. aeruginosa-C. albicans* polyspecies biofilms. (A) Left panel: aerobic condition; (B) right panel: anaerobic condition. Red boxes indicate differentially expressed proteins in comparison between control polyspecies biofilm and polyspecies biofilm treated with 40 mg/ml combined antimicrobials (20 mg/ml erythromycin:20 mg/ml nystatin). Seventy μg of protein sample were focused on 13 cm, pH 3–10 non-linear IPG drystrips, followed by 12% polyacrylamide gel electrophoresis and SYPRO Ruby staining.

Table 1 Identification of differentially expressed proteins in *P. aeruginosa-C. albicans* polyspecies biofilms treated with 40 mg/ml combined antimicrobials (20 mg/ml erythromycin:20 mg/ml nystatin).

Spot	Accession	Protein name	MW	pI	Functional category	Protein score	Fold change
		Aerobic condition					
1	Q9HT20[#]	ATP synthase subunit beta	49526	4.98	Energy metabolism	378	−1.15
2	P09591[#]	Elongation factor Tu	43684	5.23	Protein metabolism	228	−1.40
3	P53593[#]	Succinyl-CoA ligase subunit beta	41802	5.83	Carbohydrate metabolism	104	−1.84
		Anaerobic condition					
4	Q9I2V5[#]	Aconitate hydratase 2	94196	5.22	Carbohydrate metabolism	164	−1.36
5	Q9HWD2[#]	Elongation factor G1	78077	5.06	Protein metabolism	204	+1.91
6	Q9HZ71[#]	30S ribosomal protein S1	61946	4.83	Ribosomal protein production	353	+1.24
7	Q9HT18[#]	ATP synthase subunit alpha	55530	5.04	Energy metabolism	235	+1.80
8	Q9HT20[#]	ATP synthase subunit beta	49526	4.98	Energy metabolism	224	+1.33
9	Q9HXZ5[#]	Enolase	45338	5.02	Carbohydrate metabolism	104	+3.81
10	P09591[#]	Elongation factor Tu	43684	5.23	Protein metabolism	75	+5.65
11	P53593[#]	Succinyl-CoA ligase subunit beta	41802	5.83	Carbohydrate metabolism	155	−3.14
12	Q9I576[#]	4-hydroxyphenyl pyruvate dioxygenase	40002	5.10	Protein metabolism	163	+1.30

[#] Indicates proteins from *P. aeruginosa*.

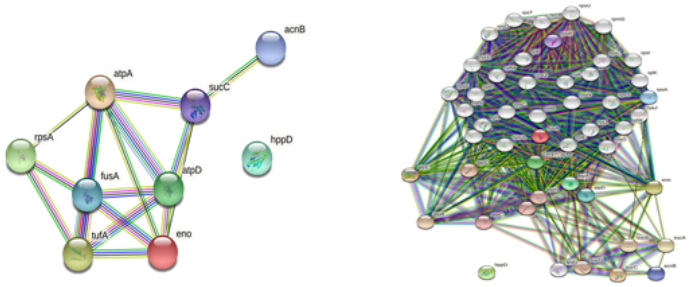
Expression level of ATP synthase subunit beta, elongation factor Tu, and succinyl-CoA ligase subunit beta

ATP synthase subunit beta, elongation factor Tu, and succinyl-CoA ligase subunit beta were found to be expressed in *P. aeruginosa* biofilm under both aerobic and anaerobic conditions (Table 1). Treatment with the combined antimicrobials resulted in changes in their expression. Both ATP synthase beta (aerobic: −1.15; anaerobic: +1.33) and elongation factor Tu (aerobic: −1.40; anaerobic: +5.65) were downregulated and upregulated under aerobic and anaerobic conditions, respectively. On the other hand, succinyl-CoA ligase subunit beta (aerobic: −1.84; anaerobic: −3.14) was downregulated under both aerobic and anaerobic conditions.

Interaction network of differentially expressed proteins in the polyspecies biofilms

Differentially expressed proteins in the polyspecies biofilm under anaerobic condition were further analyzed to understand their molecular interactions. Fig. 2A shows functional linkages between differentially expressed proteins in the polyspecies biofilm. Number of edges, average node degree, and average local clustering coefficient were computed to be 17, 3.78, and 0.707, respectively. The predicted pathways included carbohydrate metabolism, amino acid metabolism, secondary metabolites metabolism, oxidative phosphorylation, propanoate metabolism, and

A)



B)

Parameter	Network 1	Network 2	Network 3	Network 4	Network 5	Network 6
Number of node	9	14	19	29	39	49
Number of edge	17	47	94	217	443	794
Average node degree	3.78	6.71	9.89	15	22.7	32.4
Average local clustering coefficient	0.707	0.77	0.75	0.757	0.811	0.86
P value	$1.81e^{-07}$	$2.61e^{-13}$	$<1.0e^{-16}$	$<1.0e^{-16}$	$<1.0e^{-16}$	$<1.0e^{-16}$

C)

Identifier	Protein	Hub protein	Position
hppD	4HPPD: 4-hydroxyphenylpyruvate dioxygenase	No	Outlier
eno	Enolase	Yes	Center
atpD	ATP synthase subunit beta	Yes	Center
fusA1	Elongation factor G	Yes	Center
acnB	aconitate hydratase 2	No	Periphery
tufA3	Elongation factor Tu	Yes	Center
rpsA	30S ribosomal protein S1	Yes	Center
atpA	ATP synthase subunit alpha	Yes	Center
sucC	Succinate--CoA ligase	Yes	Periphery

Fig. 2 Protein interaction network using differentially expressed *P. aeruginosa* proteins in the polyspecies biofilm treated with 40 mg/ml combined antimicrobials (20 mg/ml erythromycin:20 mg/ml nystatin). (A) Left panel: without additional nodes; right panel: with additional nodes. (B) Stats of different size protein interaction network. (C) Differentially expressed proteins as hub proteins (> 10 functional linkages).

TCA cycle. To identify whether these differentially expressed proteins function as hub proteins, a total of 49 *P. aeruginosa* proteins were added to the network. Increasing the number of nodes in the protein interaction network substantially increased the number of functional linkages (Fig. 2B), leading to identification of enolase, ATP synthase subunit beta, elongation factor G, elongation factor Tu, 30S ribosomal protein S1, ATP synthase subunit alpha, and Succinate Co-A ligase as hub proteins exhibiting more than 10 functional linkages (Fig. 2C). In contrast, increasing the network size did not show any functional linkages for 4-hydroxyphenylpyruvate dioxygenase (4HPPD).

DISCUSSION

The present study focuses on a mixture between *P. aeruginosa* and *C. albicans*. This is since *P. aeruginosa* has a high tendency to interact with *C. albicans* in the polyspecies biofilms. Their interaction is known to be mediated by quorum sensing system [25]. A quorum sensing molecule, namely 3-oxo-HSL, is needed for the adherence of *P. aeruginosa* cells to *C. albicans* hyphae in the polyspecies biofilm [26] while the co-infection of *P. aeruginosa* and *C. albicans* has been well documented in cystic fibrosis patients. A previous work demonstrated that the interaction with *C. albicans* caused proteomic changes in *P. aeruginosa* biofilm, and *C. albicans* biofilm also showed altered proteome expression in response to *P. aeruginosa* biofilm [21].

Oxygen plays a crucial role in the metabolism of the microorganisms. Changes in oxygen level would often affect the amount of reactive oxygen species and expression of proteins associated with oxidative phosphorylation. In the present study, the oxygen level was found to affect the proteome expression in both control and treated polyspecies biofilms (Fig. 1). Proteomic study of single species biofilms under aerobic and anaerobic conditions has previously been

reported [18]. However, the study on the effect of oxygen level on proteome expression in the polyspecies biofilms is still limited. The finding from the present study suggests the impact of oxygen level on the proteome expression in the polyspecies biofilms.

Our previous work [24] showed that erythromycin in combination with nystatin (20 mg/ml erythromycin:20 mg/ml nystatin) was effective against the polyspecies biofilms. To understand the antibiofilm mechanism of combined antimicrobials, the present study investigates the proteome expression in the polyspecies biofilms, but not single species biofilms. This is because the combined antimicrobials have been shown to exert the strongest and synergistic effects on the polyspecies biofilms [24], and each antimicrobial should target the microorganism according to its molecular basis of inhibitory action. It has been established that erythromycin displays its bacteriostatic activity by binding to the 50S subunit of the bacterial rRNA complex while nystatin causes the death of the fungus by forming pores in the membrane that lead to K+ leakage and acidification.

There are several mass spectrometry-based approaches currently used for proteome profiling. The more established and widespread method uses high resolution, two-dimensional polyacrylamide gel electrophoresis to separate proteins mixture, followed by selection and staining of differentially expressed proteins to be identified by mass spectrometry. MALDI-TOF mass spectrometry is a versatile analytical technique to detect and characterize proteins. In this ionization method, protein samples are fixed in a crystalline matrix and are bombarded by a laser. The protein molecules vaporize into the vacuum while being ionized at the same time without fragmenting or decomposing. Ions are separated by their mass to charge ratio, and their mass to charge ratio are determined by the time it takes for the ions to reach a detector. In the present study, treatment with combined antimicrobials triggered differential proteome expression in the polyspecies biofilms under both aerobic and anaerobic conditions. Following the combined antimicrobial treatment, there were 12 *P. aeruginosa* proteins found to show differential expression in the polyspecies biofilms under both aerobic and anaerobic conditions (Table 1); however, there were no *C. albicans* biofilm proteins showing differential expression. Differential proteome expression in the single species *P. aeruginosa* biofilm following treatment with antimicrobial has previously been reported [27]. Also, Hoehamer et al [28] demonstrated that the proteome expression in *C. albicans* was altered following the treatment with azoles, polyenes, and echinocandins antifungals. Nevertheless, there is no published work available on differential proteome expression in *P. aeruginosa* and *C. albicans* in the form of polyspecies biofilms following antimicrobial treatment. Thus, the present work demonstrates the first evidence on the effect of combined antimicrobials on the proteome expression pattern in *P. aeruginosa*-*C. albicans* biofilms. On the other hand, the situation whereby *C. albicans* in the polyspecies biofilm does not show any differentially expressed proteins following treatment with nystatin remains unclear. A previous work has demonstrated unaffected protein expression in fungus exposed to heat shock and phosphate mineral [29].

ATP synthase subunit beta is a membrane-bound enzyme involved in ATP synthesis. It harnesses the energy from the proton gradient established across the inner mitochondrial membrane to drive the ATP synthesis. In the present study, ATP synthase subunit beta was downregulated and upregulated under aerobic and anaerobic conditions, respectively (Table 1). Downregulation of ATP synthase subunit alpha has been observed in *Salmonella typhimurium* biofilm treated with antimicrobial dimethyl sulfoxide under aerobic condition [17]. Meanwhile, upregulation of ATP synthase subunit beta has been observed in *P. aeruginosa* biofilm following treatment with *Chromolaena odorata* chloroform extract under anaerobic condition [18]. Differential response of ATP synthase subunit beta in *P. aeruginosa* biofilm towards erythromycin is possibly due to different oxygen level. Elongation factor Tu is a protein that functions at the ribosome during translation process. It is a highly conserved protein in bacteria and facilitates the binding of aminoacyl tRNA to the ribosome. The present study showed downregulation and upregulation of elongation factor Tu under aerobic and anaerobic conditions, respectively (Table 1). Reduced expression of elongation factor Tu in *S.* Typhimurium biofilm following treatment with an antimicrobial agent, dimethyl sulfoxide, under aerobic condition has previously been reported by Yahya et al [17]. On the other hand, increased expression of elongation factor Tu in *Streptococcus suis* biofilm treated with erythromycin has been shown by Zhao et al [30]. Collectively, ATP synthase subunit beta and elongation Factor Tu constantly show the proteomic response towards antimicrobials under aerobic and anaerobic conditions while the present study demonstrates the first evidence of such responses in the polyspecies biofilms. Considering the fact that ATP synthase subunit beta and elongation factor Tu are the most abundant proteins in many microorganisms [31], they may represent potential biomarkers for the investigation of antibiofilm mechanism.

Protein interaction network is a set of functional associations between 2 or more proteins that occur in a living system as a result of biochemical reaction. It is steered by interactions that include electrostatic forces, hydrogen bonding, and the hydrophobic effect. There are many examples of protein interaction network in microorganisms including cellular metabolism, signal transduction, oxidative phosphorylation, and mem-

brane transport. In the present study, the protein interaction network of *P. aeruginosa-C. albicans* polyspecies biofilms treated with the combined antimicrobials under anaerobic condition was constructed using STRING database. Six of the identified differentially expressed proteins in the polyspecies biofilms were found to be hub proteins (Fig. 2C). Several works have shown that antimicrobial drugs produce specific effects on gene expression profile and protein interaction network [32, 33]. Thus, investigating the protein interaction network in the biofilms treated with antimicrobials may provide an insight into the antibiofilm mechanism. It is possible that the combined antimicrobials inhibit *P. aeruginosa-C. albicans* polyspecies biofilms by interfering the functional linkages associated with carbohydrate metabolism [17, 34]. In addition, the present finding shows that most of the hub proteins are located at the centre of the protein interaction network, corroborating several previous works [17, 35–37].

CONCLUSION

Treatment with the combined antimicrobials causes proteomic changes in *P. aeruginosa-C. albicans* polyspecies biofilms under aerobic and anaerobic conditions. In response to the combined antimicrobials, *P. aeruginosa* proteins show differential proteome expression while *C. albicans* does not. It is possible that the combined antimicrobials inhibit *P. aeruginosa-C. albicans* polyspecies biofilms by interfering the protein interaction network associated with multiple biological processes. The findings from the present study may be useful to manage the antimicrobial resistance and the diseases caused by *P. aeruginosa-C. albicans* polyspecies biofilms. It is recommended that the future work should determine the transcriptomic changes in *P. aeruginosa-C. albicans* polyspecies biofilms following treatment with the combined antimicrobials.

Acknowledgements: We would like to thank all laboratory staff from Agro Biotechnology Institute, Serdang, Selangor for providing technical advice on mass spectrometry. This research was funded by Malaysian Ministry of Higher Education under a research grant, 600-IRMI/FRGS 5/3 (417/2019).

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